Comparative Gene Expression Analyses of *Campylobacter jejuni* Strains Isolated from Clinical, Environmental and Animal Sources

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ABSTRACT

Campylobacter species are the primary cause of bacterial food-borne diarrhoea worldwide. Comparative genomic analyses of *Campylobacter* strains reveal genome plasticity providing insight into the evolution of virulence traits. The goal of this study was to identify genes important for infectivity and for naturally occurring variability in phenotypic traits in *C. jejuni* and *C. coli* strains. Transcriptome and phenotype analyses were conducted to determine if genetic and phenotypic characteristics could be attributed to the source of the strains. Isolates from water sources had higher biofilm formation than animal strains. Clinical strains had decreased sensitivity to hydrogen peroxide as well as increased adherence and invasion when compared to animal strains. A number of genetic differences were observed; however, without further analysis it is difficult to determine which of these impact virulence in *Campylobacter*. Ultimately, this project will lead to the identification of markers associated with strains of *Campylobacter* causing illness.

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LIST OF ABBREVIATIONS

CAT	Chloramphenicol Acetyltransferase
CCDA	Charcoal Cefoperazone Deoxycholate Agar
cDNA	Complementary DNA
CFU	Colony Forming Units
CJIE	*Campylobacter jejuni* Integrated Element
CMLP1	*Campylobacter* Mu-Like Phage 1
CPS	Capsular polysaccharide
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia coli*
EBSS	Earle's Balanced Salt Solution
FBS	Fetal Bovine Serum
gDNA	Genomic DNA
LB	Luria Bertani
LOS	Lipooligosaccharide
MH	Mueller Hinton
MHA	Mueller Hinton Agar
NML	National Microbiology Laboratory
OD_{600}	Optical Density at 600nm
OISB	Ottawa Institute of Systems Biology
OMRB	Old Man River Basin
PHAC	Public Health Agency of Canada
PMT	Photomultiplier tube
RNA	Ribonucleic Acid
SSB	Semi-Solid Brucella
ssDNA	Single Strand DNA
WTP	Water Treatment Plant

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LIST OF TABLES USING THEIR DESCRIPTIVE TITLES

1: INTRODUCTION

1.1 *Campylobacter*

While analyzing stool samples in 1886, Theodore Escherich came across non-culturable spirally curved bacteria (138). However, *Campylobacter* were not identified until 1906 when two British veterinarians, John McFadyean and Stewart Stockman, described seeing 'large numbers of a peculiar organism' in the uterine mucus of pregnant sheep (138, 170). Later in 1913, these two surgeons completed the first isolation of *Campylobacter* from aborted bovine fetuses. Due to their spirally curved rod shape, *Campylobacter* were first described as *Vibrio* species however, in 1963, Sebald and Veron suggested the genus *Campylobacter* for these non-fermentative, microaerophilic bacteria with small, A+T rich, genomes (138, 164, 170).

1.1.1 *Campylobacter* spp.

The phylum Proteobacteria consists of five classes including the epsilon proteobacteria (52, 116, 155). The epsilon proteobacteria are commonly found in gastrointestinal tracts of animals and in the environment. In this class, the order *Campylobacterales*, that includes the *Campylobacteraceae* family, contains human and animal pathogens (52, 152, 164). The *Campylobacteraceae* family includes *Campylobacter* and *Arcobacter*, two bacteria that are commensals in humans and animals (75, 128, 138). The 18 species in the genus *Campylobacter*, including *Campylobacter jejuni* and *Campylobacter coli*, can be isolated from several sources

(22, 31, 52). While many of the thermophilic species are part of the natural gut biota of wild and domestic animals, they can be pathogenic in humans (9, 75, 128).

1.1.2 Morphology, motility, growth and speciation of *Campylobacter*

Campylobacter species are small (0.2-5μm in length and 0.2-0.9μm in width) (63, 138), Gram-negative (14, 34), microaerophilic (5-10% oxygen and 5-13% carbon dioxide) (65, 154), non-spore-forming (138), spiral-shaped bacteria (22, 57, 128) that are often linked to diarrhoeal disease in humans worldwide (31, 63, 95). Their corkscrew motility is mediated by a single unsheathed polar flagellum present at one or both ends of the cell, favoring displacement of the bacteria through the mucous layer (34, 50, 170). Flagella and the motility they confer are important virulence factors required for adhesion and invasion of *Campylobacter spp.* (11, 22, 156), leading to human infection and colonization of the animal host (14, 34, 170). The *Campylobacter* flagellar structures also serve as type III secretion systems for flagellar and non-flagellar proteins important for adhesion and invasion of host cells (34, 170). The assembly of a functional basal body, hook, and at least one filament protein is necessary for secretion (34, 170).

These oxidase- and catalase-positive bacteria (65, 66, 138) are unable to ferment or oxidize carbohydrates for energy because they do not produce the 6-phosphofructokinase enzyme important for the glycolytic cycle (112, 138, 150). *Campylobacter* metabolize amino acids and organic acids, including tricarboxylic intermediates present inside the host (65, 138, 150). Amino acids are important sources of carbon and energy, with preference given to serine, aspartate, glutamate

and proline (65, 150). *Campylobacter* metabolize sodium pyruvate for increased electron transport and ATP generation (58).

Thermophilic *Campylobacter* spp. commonly associated with human disease grow at temperatures above 30°C and below 46°C, but optimal growth temperature is between 37 and 42°C (38, 63, 128). *C. jejuni* persist outside a host but growth is limited (131, 155). Furthermore, *Campylobacter* spp. survive and proliferate at optimum pH levels between 6.5 and 7.5 (65, 138). In situations of extreme pH levels and temperatures, nutrient starvation, extreme moisture content, and osmotic shock, the morphology of *Campylobacter* cells changes from culturable spiral rods to non-culturable coccoid shapes (56, 63, 65). This latter is referred to as the Viable But Non-Culturable (VBNC) state of the bacterium, but the importance of it for human infection is still unclear (1, 56, 138).

Biochemical tests have traditionally been used to identify clinically important *Campylobacter* species (99, 124, 138). Tests for catalase production, hippurate hydrolysis, indoxyl acetate hydrolysis and growth at 42°C will enable identification of the most common clinical isolates. *C. jejuni* hydrolyses hippurate while the other catalase-positive species, *C. coli*, *C. lari* and *C. fetus*, do not. Finally, *C. jejuni, C. coli* and *C. lari* are capable of growth at 42°C, while *C. fetus* is not. Identification can also be achieved using rapid immunological test kits and PCR-based methods.

1.1.3 *Campylobacter* outer-membrane surface polysaccharide structures

C. jejuni and *C. coli* lipooligosaccharides (LOS), N-linked glycoproteins, capsular polysaccharides (CPS) and O-linked flagellar glycoproteins are important for motility, virulence, colonization, serum resistance, stress response, survival

outside the host and evasion of the host immune system (28, 92, 164). LOS is similar in structure to lipopolysaccharides (LPS), with the exception of an O-antigen polysaccharide chain in LOS (69). Molecular mimicry of *C. jejuni* and *C. coli* LOS to human gangliosides has been associated with the development of Guillain-Barré syndrome (GBS), an important cause of paralysis (63, 69, 170). Phase variation of CPS structural genes results in modifications of the CPS and influences serum resistance, adhesion and invasion to epithelial cells, and colonization of chickens (164). Two protein glycosylation systems were identified in *C. jejuni* (51, 145). The O-linked glycosylation system alters serine or threonine residues on flagellin and is important for flagellar assembly and host-cell interactions. The N-linked glycosylation system modifies asparagine residues on several proteins, but the involvement of this modification in the virulence mechanisms requires further investigation (28, 92, 164). The N-linked glycosylation system is conserved among all *C. jejuni* and *C. coli* strains but LOS, CPS and O-linked glycosylation systems vary (164).

1.1.4 Natural transformation and antibiotic resistance of *Campylobacter*

Naturally competent *Campylobacter* strains acquire and incorporate external DNA into their genome, leading to the addition or loss of genes, genomic rearrangements and genetic diversity (40, 69, 135). Horizontal gene transfer of motility genes occurs between *Campylobacter* strains *in vitro* and in the chicken caecum illustrating genome plasticity (24, 159, 160). For the uptake of free DNA from the environment, natural transformation is controlled by surface polysaccharides that interact with plasmids, chromosomal DNA and antibiotics (69,

97, 164). Strains can acquire resistance by naturally transforming DNA encoding proteins conferring resistance to a specific antibiotic (38, 53). *C. jejuni* strains favour DNA uptake from other *C. jejuni in vitro*, and natural transformation is regulated by environmental conditions such as carbon dioxide and viable bacterial cell count (80, 103, 160). N-linked protein glycosylation, LOS biosynthesis, Type II and possibly Type IV secretion system, and the DNA processing enzyme DprA, are important factors for natural transformation in *C. jejuni* (41, 164).

1.2 *Campylobacter*: A human pathogen

1.2.1 Importance of *Campylobacter* infections

According to the World Health Organization (WHO), campylobacteriosis, a gastroenteritis infection caused by *Campylobacter* spp., affects 1% of the population every year in developed countries (63, 159). An estimated 400-500 million cases of diarrhoea worldwide per year (5-14% of all cases) are caused by *Campylobacter* spp. (2, 38, 84). The primary cause of campylobacteriosis is *C. jejuni* accounting for greater than 90% of all cases in humans while *C. coli*, *C. lari* and *C. upsaliensis* are responsible for 2.5%, 0.2%, and 0.01% of cases, respectively (38, 117, 159). *Campylobacter* is a leading cause of bacterial gastroenteritis worldwide with more cases than *Salmonella*, *Escherichia coli*, *Shigella*, *Clostridium*, and *Listeria monocytogene* (2, 58, 159).

Being ubiquitous, *Campylobacter* spp. are significant contributors to food-borne illnesses with an infectious dose of as little as 500 bacterial cells (105, 107, 135). *Campylobacter* rank as the fourth most important foodborne pathogen (9% of cases) in the USA after norovirus, nontyphoidal *Salmonella* species and *Clostridium*

perfringens, respectively, which are responsible for 58%, 11% and 10% of cases (133). Close to 80% of *Campylobacter* infections are associated with contaminated food products, primarily chicken (96, 97, 159). Individuals of all ages are susceptible to *Campylobacter* infections. Children aged less than 5 years in developing countries and people between 15-39 years old in industrialized countries are affected more often (67, 139, 170). Cases of gastroenteritis caused by *Campylobacter* are often sporadic and increase in number during the summer season (9, 60, 155). *Campylobacter* outbreaks are rare but when they occur, untreated water, raw milk and chicken products are among the sources of infection (5, 106, 138).

1.2.2 Economic impact

With estimated costs of 1 747 million dollars in 2009, *Campylobacter* and *L. monocytogenes* were both ranked as third most costly pathogens in the USA, after *Salmonella* spp. and *Toxoplasma gondii* (133). *Campylobacter* species were responsible for 15% of all hospitalizations, ranking third after nontyphoidal *Salmonella* species (35%) and norovirus (26%) (133). *Campylobacter* in poultry was the most costly pathogen-food combination in the USA, with an estimated 1 257 million dollars due to infection from this source alone (133). In 2007, 65.7% of reported gastrointestinal illnesses in New Zealand, roughly 13 000 cases, were *Campylobacter* infections (149). The annual cost of *Campylobacter* infections in New Zealand was estimated to be more than $40 million (149).

1.2.3 Campylobacteriosis in humans

Following consumption of *Campylobacter*-contaminated foods or beverages, bacteria colonize the small intestine of the human host causing mucosal damage in ileum and jejunum, and proceed to the colon and rectum (50, 67, 117). Campylobacteriosis develops after bacterial adhesion and invasion of the host epithelial cells, leading to inflammation and the onset of diarrhoea (170). Severity of disease is dependent on strain pathogenicity, previous exposure, and the effectiveness of the host immune response (17, 170). Employees of poultry abattoirs develop immunity after an extended period of exposure following initial illness (22, 76). Immunocompromised patients and individuals suffering from diabetes have a greater risk of developing severe symptoms of campylobacteriosis (63, 87, 155).

1.2.3.1 Symptoms of infection

Symptoms vary with the number of days after intestinal colonization. From the first to the third day, patients complain of headaches, fever, nausea, vomiting and fatigue (31, 77, 112). Clinical symptoms including watery or bloody diarrhoea, abdominal pain, mucosal damage and inflammation are present from the third day on (75, 77, 109). Bowel movements decrease around the sixth day, but *Campylobacter* can still be isolated from stool for over two weeks following infection (67). In developing countries, where children are mostly affected, patients suffer from watery diarrhoea while those in industrialized nations present with symptoms of dysentery (87, 106, 164). Campylobacteriosis is usually self-limiting and has a duration of infection of about 10 days for immunocompetent individuals (64, 68, 120).

1.2.4 Post-infection complications

Exposure to *C. jejuni* can lead to post-infectious sequelae such as meningitis, bacteraemia, pneumonia, miscarriage, Guillain-Barré syndrome (GBS), Crohn's disease, ulcerous colitis, Miller-Fisher syndrome (MFS), post-infectious irritable bowel syndrome (PI-IBS) and reactive arthritis (2, 76, 106).

1.2.4.1 Guillain-Barré syndrome following *Campylobacter* infection

An estimated 1 in every 1000 campylobacteriosis cases leads to GBS, and is the most common infection associated with GBS (roughly 40% of GBS cases) and its variant, Miller-Fisher syndrome (27, 67, 121). Due to mimicry at the molecular level between *Campylobacter* LOS and human gangliosides, *Campylobacter*-induced antibodies target the nervous system leading to axonal degeneration of peripheral and cranial nerves (67, 76, 170). This injury to the nervous tissue causes weakness and ascending paralysis of the peripheral nervous system and is sometimes fatal (9, 96, 130). Most individuals suffering from GBS recover completely within 6 to 12 months, but about 3% of cases in developed countries lead to death (67). A rare variant of GBS, Miller-Fisher syndrome, is described as a descending paralysis (63).

1.2.4.2 *Campylobacter*-associated irritable bowel syndrome and reactive arthritis

Campylobacter, *Salmonella* and *Shigella* infections are recognized as antecedents for post-infectious irritable bowel syndrome (PI-IBS) (22, 140, 170) , a disorder that manifests itself about 6 months after enteritis, as abdominal pain and frequent bowel movements (90, 140, 170). Close to 25% of PI-IBS cases are linked

to previous *Campylobacter* infections (76, 90, 140), especially in individuals where gastroenteritis persisted for more than 7 days (76). It is unclear if *Campylobacter* infection cause IBS or IBS increases the likelihood of a *Campylobacter* infection (76).

After 4 weeks of a *Campylobacter* infection, joint and muscle inflammation, known as reactive arthritis or Reiter's syndrome (76, 106), develop for some patients (2, 123). Population studies indicate that 4.3 individuals per 100 000 *Campylobacter* infections acquire reactive arthritis (76).

1.2.5 Models for human *Campylobacter* infection

Campylobacter infection studies have been performed with human volunteers, non-human primates, ferrets, mice, pigs and chickens (11, 67, 106).

1.2.5.1 *Homo sapiens* and human intestinal cell lines

In 1988, Black *et al.* (11) performed a human study by orally administering *C. jejuni* to 111 human volunteers. They determined the infection dose of this bacterium and found that the severity of disease is strain dependent, as symptoms of strain A3249 were mild while those of strain 81-176 were severe (11). While human volunteer studies are the best model for studying *Campylobacter* infection, this is usually not feasible. Human epithelial cell lines, such as Caco-2 (American Type Culture Collection, HTB 37) for example, are commonly used for *Campylobacter* cytokine, adhesion and invasion assays with different strains (67, 164).

1.2.5.2 Primate, ferret, murine and rabbit models

The pig-tailed macaque (*Macaca nemestrina*) is one of the best models of human *Campylobacter* infection (67, 106). Following oral challenge of infant primates with *Campylobacter*, they not only display symptoms of infection, including vomiting and bloody diarrhoea, but also, in most cases suffer from bacteraemia (67, 106, 164). These animals are no longer used for these types of studies for both ethical reasons and elevated caretaking expenses. Ferrets show human symptoms of diarrhoea and inflammation following oral administration of *C. jejuni* but they are expensive (67, 106, 164). Most ferrets are naturally colonized with *Campylobacter*, making it difficult to study host response. Several breeds of mice were tested in distinct experimental conditions with varying results (67, 106, 164). Murine *Campylobacter* models include, among others, the IL-10 gene-deleted mice and the humanized mouse model (7, 67, 89). IL-10 is an anti-inflammatory cytokine and in IL-10 deficient mice, inflammation of the cecum and colon was observed when mice were orally challenged with *C. jejuni* NCTC11168 (7, 89). The humanized mouse model consists of gnotobiotic mice (germfree) where the murine intestinal flora was replaced by the human gut biota (7). This model no only has an immune response comparable to the human host but also illustrates that host resistance to *C. jejuni* infection is influenced by the gut microflora composition (7). The rabbit model does not show symptoms of disease following oral inoculation but colonization with *Campylobacter* is observed (106). The Removable Intestinal Tie Adult Rabbit Diarrhoea (RITARD) and the Rabbit Ileal Loop Test (RILT) models have also been developed, but making conclusions for human infection from experiments with these surgically modified animals is difficult (67, 106).

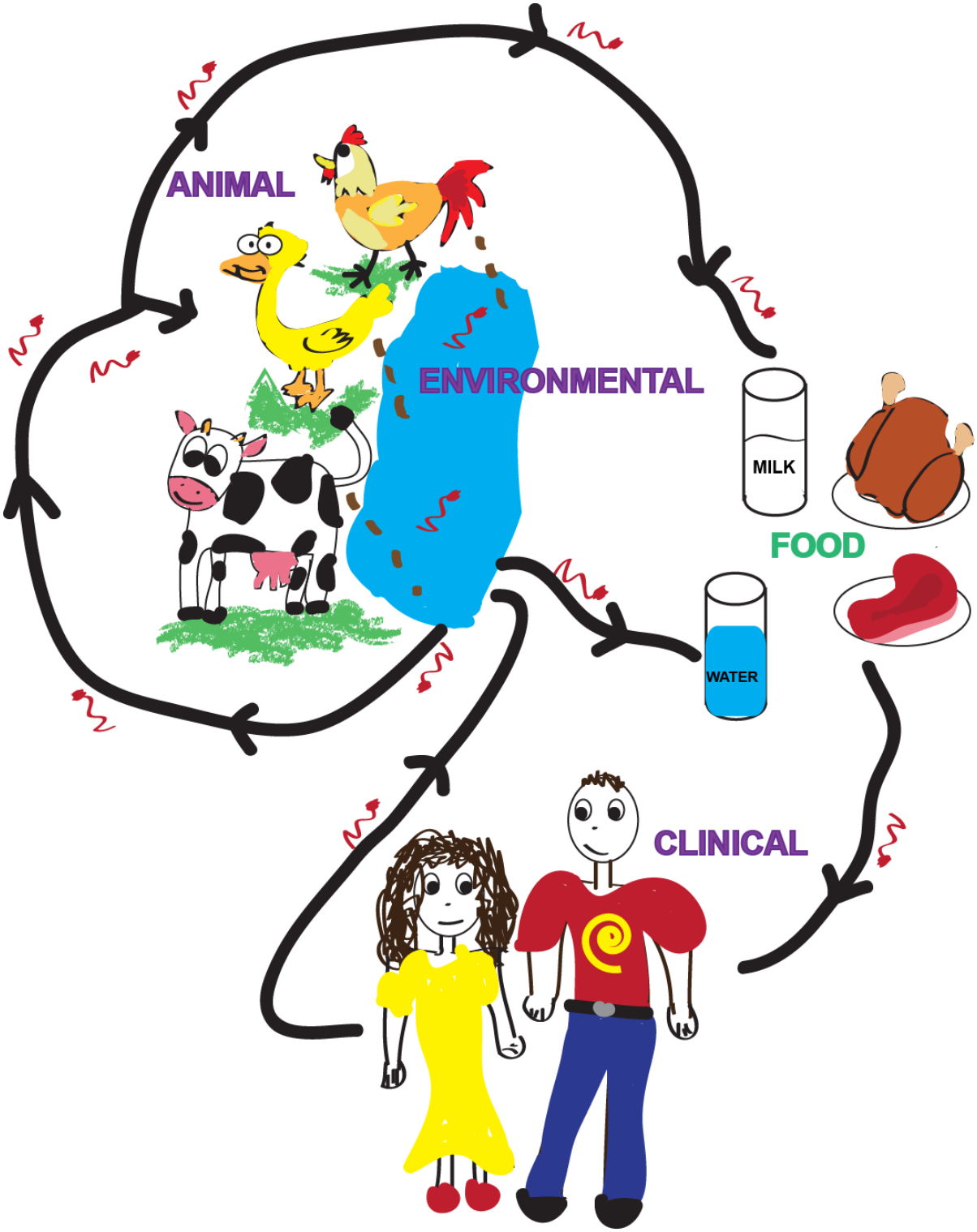
1.2.5.3 Swine and chicken models

Oral administration of *C. jejuni* and *C. coli* to neonatal piglets causes diarrhoea with bloody and mucoid stools for about 6 days. Piglets are used as models of human disease for *Campylobacter* enteritis as the human and pig gastrointestinal tracts are comparable (106, 141). Chicken is a model for *Campylobacter* colonization of the avian gastrointestinal tract and is often used to evaluate the effect of gene mutation on chicken colonization (106, 164). Although it is not clear that chicken colonization adequately reflects human infection, poultry is the primary cause of campylobacteriosis and reducing the *Campylobacter* cell counts in chickens would be beneficial to humans (67, 106).

1.3 Sources of *Campylobacter*

Campylobacter bacteria are found in the environment and in animals (Figure 1). Common sources of human *Campylobacter* infections are raw meats and poultry, untreated water and unpasteurized milk (5, 77, 121). With a high prevalence of commensal bacteria in avian species, the primary source of *Campylobacter* appears to be handling and consumption of undercooked poultry meats (112, 121, 123). In Canada, between 1996 and 2005, 56% of the 32 *Campylobacter* outbreaks were associated with contaminated poultry meats and 22% to dairy products other than milk (126).

Figure 1. Sources of *Campylobacter*. Animal, environmental and clinical sources of *Campylobacter* species contribute to human infection. *Campylobacter*, ubiquitously present in the environment, can be isolated from water and soil samples. Fecal droppings of animals commensally carrying the bacteria contaminate the environment and contribute to transmission between animals through the fecal-oral route. Consumption of unpasteurized milk, contaminated meat products and unsanitary drinking water can lead to *Campylobacter* infection in humans. In turn, human practices increase dissemination of bacteria between animals and in the environment.


ANIMAL
ENVIRONMENTAL
MILK
FOOD
WATER
CLINICAL

1.3.1 Animal reservoirs of *Campylobacter*

Campylobacter can colonize poultry, swine, cattle, sheep, wild birds, rabbits, other wild mammals, domestic animals and molluscs (31, 38, 159).

1.3.1.1 Avian species

The warm protein-rich avian body of the chicken is favoured by thermotolerant *Campylobacter* spp., and because poultry is low cost and high in demand human exposure to these bacteria is inevitable (96, 112, 138). With an infection dose of 100 bacterial cells in chicken, *C. jejuni* is the species most often isolated from poultry and it colonizes the intestine at levels up to 10^9 colony-forming units per gram (CFU/g) of faeces (34, 56, 60). The most common sites of colonization are the colon and the mucus layer of the ceca two glands located between the small and large intestine, from which the levels of *Campylobacter* are between 10^6 and 10^8 CFU/g of faeces (56, 60, 149). In birds, the presence of *C. jejuni* triggers the release of antibodies, but *C. jejuni* remains as a commensal (10, 38, 96). *C. lari* species are often found in seagulls while high numbers of *C. jejuni* cells can be found in Canada geese, migratory ducks and sandhill cranes (1, 38, 63).

1.3.1.1.1 Transmission of *Campylobacter* to poultry

Avian hosts provide optimal growth temperature of 42°C for *Campylobacter* species and the ability of this microorganism to survive outside the host encourages recurrent transmission of these bacteria from the environment to the animals (75, 131, 155). Horizontal transmission within the chicken farm environment allows for

the spreading of *Campylobacter* spp. among and within poultry flocks (14, 138). A study by Messens *et al.* (93) showed that *C. jejuni* strains recovered from the nipple water dispenser and the feed pan matched those obtained from the animal (60, 63, 155). Similar results were shown during the isolation of genetically identical *Campylobacter* spp. from dairy cows and nearby groundwater sources (63, 105, 155). In the poultry house environment, chickens are exposed to and consume faeces containing *C. jejuni*, which encourages transmission of bacteria between chickens (60, 155). Other factors influencing colonization of avian hosts with *Campylobacter* spp. include high flock size, age of flock, season, feeding process, contact with faecal matter, nearby water sources and drinking water, insects, rodents, wild birds, thinning, slaughtering and transportation of chickens, farm equipment, and abattoir workers (60, 63, 138).

1.3.1.2 Cattle, swine and domestic animals

With a prevalence of up to 80% in the intestine of dairy cows and beef cattle, *Campylobacter* species are common components of animal faecal droppings, which are frequent contaminants of raw milk during the milking process (63, 105, 138). Bacterial isolates from bovine faeces have the potential to colonize chickens contributing to the spread of *Campylobacter* species and to the risk of human infection (105). Larger numbers of bacteria have been recovered from dressed pigs when compared to beef or sheep carcasses and 84% of *Campylobacter* species extracted from pigs are *C. coli* (38, 60, 138). *C. upsaliensis* and *C. lari* recovered from household pets, such as cats and dogs, have been directly associated with human *Campylobacter* infections (31, 38, 63). A correlation was found between

summer peaks in human *Campylobacter* infection and the canine breeding season (63).

1.3.2 Environmental sources of *Campylobacter*

1.3.2.1 Transmission of *Campylobacter* from animals and humans into the environment

The transmission of *Campylobacter* from the hosts to the environment is thought to occur through farm runoffs or faecal deposits from animals, birds or infected humans (2, 21, 84). Animals are important factors in the spread of this bacterium in the environment (Figure 1). Wild birds such as the shoveler duck feed by digging for shellfish in water sediments, where *Campylobacter* species are often found (1, 139, 166). During migration, wild birds carrying this bacterium in their gut transmit these microorganisms to new regions (63). The dissemination of bacteria occurs through animal faeces coming in contact with nearby bodies of water or with other livestock (1, 84). *C. jejuni* have been found in sewage water and fresh water sources, including rivers, lakes, ponds and groundwater, all of which contain wastes from poultry, wild birds, and dairy animals (10, 74, 159). Prevalence of *Campylobacter* bacteria isolated from rivers, lakes, ponds and coastal seas is higher during the fall, winter and early spring seasons while the levels of *Campylobacter* spp. in sewage water numbers increase during the summer (1, 84). The use of contaminated waters for recreational activities, on the farm and in the household, increases human exposure to *Campylobacter* (1, 139). Contact with water and sources other than food are associated with 20% of *C. jejuni* human infections (2, 21, 84).

1.3.2.2 Survival of *Campylobacter* in bodies of water

The length of bacterial survival in aquatic environments is dependent on nutrient levels and predation but is increased in biofilms, when present inside protozoa, lower temperatures and lack of sunlight (UV rays) (1, 139). The relationship between protozoa and *Campylobacter* species in the water environment is still uncertain. Protozoa, like amoebae for example, internalize and do not digest *Campylobacter*, but it is unclear if they are protecting and allowing the organism to multiply in the water (84, 139). The protozoan hosts for *C. jejuni* and *C. coli* include *Acanthamoeba castellanii* and *Tetrahymena pyriformis* (56, 139). These hosts protect the bacteria from disinfectants and encourage survival at varying temperatures in the broiler house and in the drinking water systems (139). Studies demonstrate protozoa helping *Campylobacter* to survive in natural biofilms by preying on other microorganisms and consequently increasing nutrient availability (84, 139). This exchange between biofilms, protozoa and *Campylobacter* can also possibly protect the bacteria during water treatment processes (139).

1.3.3 Clinical sources of *Campylobacter*

1.3.3.1 Transmission of *Campylobacter* from food and the environment to humans

The spread of *Campylobacter* through person-to-person contact does occur via the faecal-oral route, but human infection is more likely to be caused by oral exposure to contaminated poultry, water or raw milk (60, 97, 107). Livestock and environmental sources are important reservoirs of *Campylobacter* spp. These bacteria are transmitted to humans through consumption of undercooked poultry,

contaminated meat products, unpasteurized raw milk and unsanitary drinking water (Figure 1). Food handling also poses a threat, particularly with cross-contamination in the kitchen (60). Environmental and recreational water sources are also good vehicles for human infection by *Campylobacter* spp., not only through direct exposure, but also by contaminating filter feeders such as shellfish (138).

1.3.3.2 Pathogenesis in humans

While human acidic stomach conditions may limit the survival of bacteria ingested, some neutralizing food can be protective (67, 140). Up to 10^6 CFU/mL and 10^{13} CFU/mL of bacteria can be found in the ileum and cecum, respectively (67, 140). Bacterial cells adhere to epithelial cells or the mucus lining of the intestine where they multiply to either colonize asymptomatically or cause gastroenteritis. The outcome of infection is dependent on the human immune response against *Campylobacter* (67, 76). Following frequent exposure to similar serotypes, the immune response causes an asymptomatic infection as was the case for children in undeveloped countries (67, 76). *Campylobacter* either adhere to intestinal cells, and then produce toxins that inhibit intestinal fluid absorption to cause secretory diarrhoea, or the bacteria invade and multiply in the mucosal lining of the intestine leading to inflammatory response and dysentery (67).

1.3.3.2.1 Interaction of *Campylobacter* with epithelial cells

In the human host, toll-like receptors (TLRs) are important for pathogen recognition because of their ability to identify patterns on bacterial cells (67). Toll-like receptors TLR4, TLR5 and TLR9 are not activated against *Campylobacter* (67). The

nucleotide-binding oligomerization domain-containing protein 1 (NOD1) receptor and natural resistance-associated macrophage protein (NRAMP) contribute to innate immunity against *Campylobacter* and the development of inflammatory disease (28, 50, 67). *Campylobacter* flagellin, major outer membrane proteins, capsular polysaccharides and the cytolethal-distending toxin (CDT) serve as antigens eliciting immune responses in the human host (67).

1.3.3.2.2 Human immune response

Symptoms of gastroenteritis including inflammation and diarrhoea occur after adhesion to the mucosal lining and invasion of host epithelial cells (78, 79, 96). The majority of proinflammatory responses to *Campylobacter* are dependent on the presence of NF-κB and activator protein 1 (AP-1) (67, 164). In the presence of *Campylobacter*, the innate and cell-mediated immune responses are triggered, leading to dendritic cells maturation and the release of proinflammatory cytokines (67, 140, 164).

1.3.3.2.3 Treatment of *Campylobacter* infection

The majority of *Campylobacter* infections are self-limiting illnesses requiring replenishment of fluids and electrolytes (67, 138). Patients with severe or recurrent infections are treated with antibiotics such as macrolides, tetracycline and fluoroquinolones (67, 69, 138). Both *C. coli* and *C. jejuni* are developing increasing resistance to these medications. Phase variation of *Campylobacter* LOS interferes with the development of an effective vaccine, thus researchers are focusing on the *Campylobacter* capsule (76).

1.3.3.2.4 Virulence properties

Several properties of *Campylobacter* have been shown to enhance the ability of the bacteria to cause disease in humans. These include flagellar motility, adherence, invasion and toxin production (79, 125, 159).

1.3.3.2.4.1 Flagella, motility and chemotaxis

Campylobacter flagella and motility are important contributors to virulence by allowing bacteria to adhere to and invade epithelial cells (34, 79, 164). Flagellin is required for colonization in the host and it plays a key role in the activation of the immune response against *Campylobacter* (14). *Campylobacter* motility is dependent on flagellar assembly and chemotaxis, both regulated by two-component signalling systems (72). Chemotaxis in *Campylobacter* steer the bacteria towards environments permitting colonization (75).

1.3.3.2.4.2 Cytolethal-distending toxin

The cytolethal-distending toxin (CDT) expands the cytoplasm of eukaryotic cells (22, 170). CDT causes eukaryotic cell death, after approximately 48 h, by blocking CDC2 kinase, interrupting the cell cycle in the G2/M phase and stopping the cells from undergoing mitosis (22, 65, 138). CDT is expressed in chicken, but does not produce an inflammatory response (22, 121, 155). The three subunits, CdtA, CdtB and CdtC are all required for adequate functioning of this protein and IL-8 secretion in humans (121, 138, 170). While CdtA and CdtC subunits bind to the host cell and transport the active subunit CdtB into the cell, the DNase I homolog CdtB is responsible for damaging host DNA (65, 138, 170). CDT is present in *C.*

jejuni, *C. coli* and *C. fetus*, and is currently the only *Campylobacter* toxin characterized (22, 31, 155). CDT, when expressed, contributes to virulence in *Campylobacter* spp. (22, 65, 138) however it is not always required for pathogenicity of the bacteria (22, 65, 100). Not all clinical strains possess CDT activity, and, in a study by Mortensen *et al.* (100), absence of this activity did not result in a decreased severity of campylobacteriosis infection in humans (100).

1.4 *Campylobacter* genome

In 2000, the first *Campylobacter* whole genome sequence was published for strain *C. jejuni* NCTC11168, a human clinical isolate (116). This genome consists of a single 1.6 megabase pairs (Mb) circular chromosome, with a 30% GC content that codes for 1643 proteins (1654 prior to re-annotation) and 54 stable RNA species (28, 116, 121). Hypervariable sequences containing homopolymeric G:C tracts are present in regions encoding proteins responsible for biosynthesis of surface structures including the capsule, LOS and flagellum (87, 116, 164). Modifications to the genome sequences such as phase variation, gene duplication and deletion, as well as frameshifts and points mutations, lead to changes in these tracts (116, 164). Several strains have now been sequenced including the *C. jejuni* RM1221, a chicken isolate containing four integrated elements not found in strain NCTC11168 (52, 59, 116). In the genus *Campylobacter*, genomes range from 29.5-44.5% in GC content, with 1.5-2.1 Mb and 1425-1931 open reading frames (31). With the increased sequence information available, a pan-genome for *Campylobacter* species was elaborated showing the core genes, those present in at least 95% of the strains, and the dispensable genes (46, 62, 80). The pan-genome of *C. jejuni*

and *C. coli* consists of approximately 3000 genes with 1317 being core genes (80). The pan-genome is altered by genetic transfers between species of *Campylobacter* and uptake of DNA from the surroundings, be it from the host or the environment (80).

1.4.1 Genome plasticity

Among the 22 variable regions in the *C. jejuni* genome, the regions showing the most differences are those important for biosynthesis of surface carbohydrate structures including LOS, polysaccharide capsule and O-linked glycosylation of flagellin (121, 135). In these regions, gene expression and protein expression are respectively regulated by slipstrand mispairing in homopolymeric GC tracts and phase variation (65, 135, 160). Natural transformation, requiring type II secretion systems, as well as slip strand mispairing and phase variation permit rapid adaptation and survival in different hosts and environments (135, 157, 159). Horizontal gene transfer, recombination and mobile genetic elements such as transposons, plasmids and phage insertions influence the evolution and pulsed-field gel electrophoresis (PFGE) patterns of *Campylobacter* strains (5, 38, 157). Intraspecies genetic recombination occurs often *in vivo* between *Campylobacter* strains leading to the emergence of heterogenic genotypes (134, 157).

1.4.1.1 Mobile genetic elements

Mobile genetic elements are DNA sequences encoding proteins important for movement of DNA in and between bacterial cells (39). In prokaryotes, mobile genetic elements include plasmids, bacteriophages and transposons (39).

1.4.1.1.1 Phage insertions in *Campylobacter*

In 1968, the first extraction of lysogenic or temperate bacteriophages was done from *C. fetus* and these mobile genetic elements were shown to complete horizontal DNA transfer of genes important for virulence in the bacteria (20, 38). Uptake of bacteriophages into *C. jejuni* causes differences in the genome structure and phenotype (5, 135). *C. jejuni* RM1221, a chicken isolate, has three bacteriophage-like elements including *Campylobacter* Mu-Like Phage I (CMLP1) and two *C. jejuni* Integrated Elements (CJIE2 and CJIE4). Based on the genomic sequence of this strain, CMPL1 serves as a Mu-like bacteriophage, while the integrated elements encode genes associated with bacteriophages (5, 40, 68). CMLP1, otherwise called CJIE1, is commonly found among *C. jejuni* strains that are not naturally transformable (41, 68). Among the 32 genes present on CJIE1, 16 encode proteins similar to bacteriophage Mu and Mu-like prophage, 12 are hypothetical proteins, 3 are conserved domain proteins and the *dns* (CJE0256) gene, an extracellular DNase, correlates with *C. jejuni* strains having a decreased natural transformation ability (41, 68). CJIE2 and CJIE4 contain DNA/RNA nonspecific endonucleases encoded by *CJE0566* and *CJE1441*, respectively, and these contribute to the observed decrease in natural transformation of *C. jejuni* strains (68).

1.4.1.2 Conserved genes

The *C. jejuni* NCTC11168 genome consists of three sigma factors σ^{28}, σ^{54}, σ^{70} encoded by *fliA*, *rpoN*, and *rpoD*, respectively (22, 50, 87). The σ^{28} and σ^{54} sigma factors regulate flagellar genes, while σ^{70} is important for expression of

housekeeping genes (50, 72, 87). Sigma factors initiate transcription in the appropriate order for assembly of the flagella (14, 34). Other genes coding proteins important for virulence such as the cytolethal-distending toxin as well as adhesins including CadF, JlpA, PEB1, and CiaB (*Campylobacter* invasion antigen), are also conserved (31, 117, 120). Energy metabolism, cell division, protein and peptide secretion as well as genes for synthesis of DNA, RNA and proteins are also conserved (117).

1.4.1.3 Genes in plasticity regions

In a 2003 study, Pearson *et al.* (117) compared 18 *C. jejuni* strains and discovered groups of variable genes, denoted as plasticity regions (PR), which are not present in all strains. There are seven plasticity regions with a similar GC content to the remainder of the genome (59, 68, 117). Plasticity region 1 (PR1) consists of genes important for electron acceptors during respiration (31, 117). Genes of the PR2, PR3 and PR7 loci play roles in phenotypic traits and the survival of the bacteria in varying conditions (31, 68, 117). PR4, PR5 and PR6 encode genes important for antigenic surface structures including flagella, LOS and the capsule (31, 117). PR4 has sequences encoding N-acetyl neuraminic acid synthase genes *neuA1, neuB1* and *neuC1*, which are important in LOS sialylation and the development of GBS (116, 117). LOS biosynthesis genes and those involved in post-translational modification of flagellin are all present in PR5, while PR6 consists of the capsular biosynthesis locus (*Cj1415c* to *Cj1442c*) (117).

1.4.1.4 Differences in gene content of strains from varied sources

A multilocus sequence typing (MLST) comparison study found that water and wildlife isolates are related to non-livestock isolates and genetically separated from clusters of animal and clinical strains (15, 59). Comparative genomics hybridization (CGH) of 111 isolates from human, animal and environmental sources shows strain separation into 'livestock' and 'non-livestock associated' clades (15, 31, 59). However, a large proportion of clinical strains are phylogenomically related to non-livestock strains leading to the conclusion that environmental isolates may be important sources of human infection (15, 31).

1.5 Relationship between genotype and phenotype in *Campylobacter*

Virulence factors, including flagella-mediated motility, adherence, invasion and toxin production, are encoded by the core genome, but variably expressed dispensable genes encode the capsule, LOS and flagella (87, 120, 138). All of the expressed genes contribute to the survival potential of strains in varied environments (87, 170).

1.5.1 Auto-agglutination

The nonspecific clumping of *Campylobacter* cells on surfaces is an important step for the development of micro-colonies and biofilms (50). The auto-agglutination process is dependent on functional flagella and the glycans that constitute them (50). The differential expression of surface structures including the flagella, LOS and capsule appear to be responsible for differences in auto-agglutination potential of strains (50).

1.5.2 Temperature

Thermotolerant *Campylobacter* spp. grow at temperatures above 30°C and below 46°C, with preferences for temperatures between 37 and 42°C (65, 128, 142). The lack of cold shock protein genes in *Campylobacter* plays a role in bacterial adaptation at growth temperatures below 30°C (120, 138). Chemotactic responses of *C. jejuni* occur at 4°C, possibly guiding the bacteria away from stressful environmental conditions and promoting persistence (22, 63). Survival of *Campylobacter* spp. with shifting temperatures is important for virulence of the bacteria (75, 120). Stintzi *et al*. (142) determined that switching the bacteria from 37°C to 42°C environments led to the up-regulation of genes required for energy metabolism, composition of the cell wall, and transport proteins.

1.5.3 Adhesion and invasion

Several genes are important for adhesion and invasion of *Campylobacter* cells. *Campylobacter* colonization of the intestinal mucus layer is promoted by the binding of the bacteria to epithelial cells in the presence of several adhesins including JlpA, CadF, Peb1 and CapA (68, 77, 121). JlpA, a lipoprotein at the surface of the membrane, interacts with the Hsp90α heat shock protein on Hep-2 epithelial cells to stimulate NF-κB and p38 mitogen-actived protein kinase (MAPK) (77, 121). CadF triggers the release of Rac1 and Cdc43 in INT407 cells and attaches to fibronectin molecules in the host cell matrix (77, 120, 121). Adherence and invasion of Caco-2 cells *in vitro* requires the putative autotransporter CapA while CiaB is important for invasion of *Campylobacter* into epithelial cells (77, 120). Another protein thought to play a role in invasion of both INT-407 and Caco-2 cells is

the putative *Campylobacter* invasion protein CipA (*Cj0685*) (68). Both adhesion and invasion mechanisms require flagella and motility (14, 34, 50).

1.5.4 Motility

Motility in *Campylobacter*, regulated by approximately 40 genes (14, 72), plays an important role in virulence of the bacteria particularly in the invasion of epithelial cells and colonization of the chicken gastrointestinal tract (50, 87). Flagella are important for survival in varying environments because they enable bacteria to move in response to detected differences in chemotactic conditions (22, 55, 138). *Campylobacter* flagellar assembly and chemotaxis are regulated by the σ^{54}-FlgSR two-component system consisting of the sensor FlgS and the response regulator FlgR (22, 72, 138). The expression of these components is regulated by phase variation (31, 72, 87). This system is thought to be important for the secretion of non-flagellar proteins (Cia, FlaC and FspA) from the O-linked glycosylated flagellin which is believed to act as a type III secretory system, the only one in *Campylobacter* (17, 34, 138). The *CiaB* gene mutation stops the secretion of all Cia proteins, leading to decreased invasion potential of *Campylobacter* and less severe disease outcomes (17, 34, 55). FspA protein, consisting of FspA1 and FspA2, is secreted from the flagellum in *Campylobacter* and FspA2 is capable of *in vitro* attachment to host epithelial cells to induce apoptosis (17, 50, 121). FlaC protein, not a component of the flagellar apparatus, binds eukaryotic cells and promotes invasion of *Campylobacter* strains (121).

1.5.5 Biofilm formation and environmental survival

Biofilms are layers of bacteria bound to a surface surrounded by an extracellular polymeric substance (EPS) matrix providing nutrients, extracellular enzymes, DNA as well as protection from external stressors (71, 84, 139). Formation of biofilms includes initial movement of planktonic bacteria to the surface, adherence and microcolonies formation as well as production of bacterial communities inside a mature biofilm (71, 74, 128). Biofilms offer opportunities for sharing of genetic material, exopolymers, nutrients, enzymes and secondary metabolites between bacterial cells (56, 149). Understanding biofilm formation in water sources and other environmental reservoirs is possible with the ability of *Campylobacter* to survive in biofilms on inert surfaces and in various conditions (50, 84). *Campylobacter* biofilm formation requires functional flagella (50). Inactivation of the *flaA*, *flaB*, *fliA* and *flhA* genes caused a delay in pellicle formation while the *flaG* mutant is unable to form a pellicle (74, 116). Proteins involved in the motility complex including the flagellin structural proteins, hook, and basal body proteins, the filament cap protein, and the chemotaxis protein CheA, all have higher expression in biofilms (74, 128). Genes important for the general and oxidative stress response have increased expression in biofilms (74).

1.5.6 Hydrogen peroxide and stress response

The oxidative stress response is important for the microaerophilic *Campylobacter* because it helps to remove reactive oxygen species (ROS), including superoxide radicals, hydrogen peroxide (H_2O_2) and hydroxyl radicals (74, 113). *Campylobacter* aerobic respiration leads to the production of superoxide

radicals as well as H_2O_2 and, in the presence of ferrous iron, an electron is transferred to H_2O_2 producing a hydroxyl radical (113). Several *Campylobacter* genes are involved in oxidative stress defence. These include a superoxide dismutase SodB, a catalase KatA, an alkyl-hydroxyperoxidase AhpC, a ferritin Cft, a ferredoxin FdxA and a DNA-binding protein Dps (57, 113). AhpC and SodB are necessary for survival of *Campylobacter* in aerobic conditions, the absence of *cft* and *dps* genes increases sensitivity to H_2O_2, and the *fdxA* mutant is less aerotolerant than the parent strain (64, 113). Ferritin (Cft) is important for iron storage and growth of *Campylobacter* in iron-limiting conditions, while Dps is involved in oxidative stress defence by sequestering free iron to prevent production of ROS (64, 152). The first defence against exposure to air is SodB, which activates the dismutation of superoxide anion to H_2O_2 and oxygen, while KatA reduces H_2O_2 to water and oxygen and AhpC reduces organic hydroperoxide to hydrogen peroxide (102, 116). Resistance to ROS and adaptation to iron limitation is important for the colonization of the gastrointestinal tract, survival in macrophages and persistence in varying environmental conditions (64, 70).

1.5.7 Gene content and gene expression studies

Strains with similar gene content do not always display similar phenotypes because of differences in gene expression.

In a study using two genetically identical *C. jejuni* NCTC11168 strains, V1 and V26, several differences were observed when comparing the ATCC acquired strain (V1) with the laboratory-passaged strain (V26) since its isolation in 1977 (14, 34, 87). Transcript profiles of these two strains differed significantly, with V1 having

higher expression levels of flagellar and virulence associated genes. V26 flagella were scarce and this passaged strain was a poor colonizer of poultry when compared to the more motile V1 strain, which was a strong colonizer and had bipolar flagella. As expected, V1 was significantly more invasive than V26 (14, 34, 87).

Using macrorestriction profiling pulsed-field gel electrophoresis (MRP-PFGE), multi-locus sequence typing (MLST) and comparative genome hybridization (CGH) analyses, a Chicken (CS) and a Turkey isolate were found genetically identical, but their phenotypes differed (31, 57, 79). Using the piglet model, the Turkey strain was found to be more virulent, eliciting a higher degree of disease with the development of diarrhoea and degenerative lesions (31, 57, 79). The Turkey strain was more motile, more invasive in INT407 cells and secreted virulence proteins while the CS strain did not. Gene expression profiles varied between isolates with class II flagellar genes having a higher expression in the more pathogenic Turkey strain. The class II flagellar genes were important for the assembly of a functional flagellum and the decreased expression of these genes in the CS isolate explained the lack of motility in this strain (31, 57, 79).

1.6 Objectives and purpose

Comparative genomic analyses of *C. jejuni* and *C. coli* strains reveals genome plasticity and provides insight into the evolution of virulence traits in these organisms. Survival in different environments depends on gene content and gene expression in response to environmental signals. The objective of this project was to determine if naturally occurring variability in phenotypic traits among clinical,

environmental and animal *C. jejuni* and *C. coli* isolates could be linked to the source of the strains. Phenotype analysis and transcript profile experiments were conducted to characterize naturally occurring variability among isolates. It was hypothesized that environmental isolates would display a decreased expression of genetic and phenotypic pathogenic properties when compared to clinical strains. While most water strains would be expected to originate from animal sources, selection pressure for survival in the environment may lead to loss of characteristics associated with infectivity. If this is the case, there may be a subset of *Campylobacter* strains that are not a human health risk. The identification of genetic markers that distinguish non-infectious strains from clinically important *C. jejuni* and *C. coli* strains will be essential in the development of control measures for this pervasive organism.

2: MATERIALS AND METHODS

2.1 Bacterial strains

2.1.1 Phage-containing *C. jejuni* isolates

This study included three isolates (00-2425, 00-2538 and 00-2544) containing a prophage homologous to *Campylobacter* Mu-Like Phage 1 (CMLP1 or CJIE1) of strain RM1221 and a control strain with similar gene content, but devoid of phage (00-2426). These isolates from the Walkerton water-borne *E. coli* and *Campylobacter* outbreak were kindly provided by Dr. Clifford Clark (National Microbiology Laboratory, PHAC, Winnipeg, Manitoba).

2.1.2 Clinical, environmental and animal *Campylobacter* strains

Twenty-four clinical, 19 water and 24 animal *Campylobacter* isolates collected between 2004 and 2007 from the Alberta region, with a small number of isolates from other Canadian provinces (Table A1), were provided by Dr. Eduardo Taboada (Laboratory for Foodborne Zoonoses, PHAC, Lethbridge, Alberta, Canada).

2.1.3 Strains for the mutant construction

For the construction of *C. jejuni* knockout mutants, plasmids were propagated in *E. coli* DH5α (8). *E. coli* DH5α strains containing either pUC19 or pRY111, respectively, were used for the isolation of pUC19, and the amplification of the chloramphenicol acetyltransferase (CAT) gene that was required for the disruption of the *C. jejuni* gene of interest. All recombinant plasmids disrupted with the CAT

cassette were naturally transformed into *C. jejuni* NCTC11168 strain (ATCC 700819) to produce the knockout mutants.

2.2 Culturing conditions and strain maintenance

2.2.1 *Campylobacter* strains

Campylobacter strains from frozen stock were grown at 37°C under microaerophilic (5% O_2, 10% CO_2, 85% N_2) conditions in a HeraCell 150i Tri-Gas Incubator (Thermo Scientific, Ottawa, ON) on Mueller-Hinton Agar (MHA; Oxoid) plates for two days. After revival, bacteria were maintained in Semi-Solid Brucella (SSB) medium (Becton Dickinson, Mississauga, ON). When required, strains were taken from SSB medium and grown on MHA plates for 24 h prior to every assay. Strains were kept in SSB medium for a maximum of 2 weeks.

2.2.2 *Escherichia coli* strains

Escherichia coli strains were cultured in Luria-Bertani (LB) broth (Becton Dickinson) or on LB agar plates (Becton Dickinson) at 37°C in a MaxQ 6000 Incubator (Thermo Scientific). When necessary, the LB medium was supplemented with chloramphenicol or ampicillin at concentrations of 10 µg/mL and 100 µg/mL, respectively.

2.3 Phenotypic assays

Strains from clinical, environmental and animal sources were first compared using phenotypic assays to assess their pathogenic potential. These experiments included growth curves, motility, biofilm formation, hydrogen peroxide sensitivity as well as adhesion and invasion assays. Furthermore, the phage-containing *C. jejuni*

isolates and the control strain were tested for their motility, their ability to form biofilms and their sensitivity to hydrogen peroxide.

2.3.1 Growth curve assay

Strains were grown overnight on MHA plates followed by incubation for 24 h in 4 mL MH broth. Samples were then diluted to an optical density at 600 nm (OD_{600}) of 0.4 (approximately $1x10^{10}$ CFU/mL) before undergoing a 10-fold dilution series to reach $1x10^{8}$ CFU/mL. A 2 mL volume of this stock was added to a Corning T-Spinner Flask (Fisher Scientific, Ottawa, ON) in a final volume of 200 mL to obtain an initial level of $1x10^{6}$ CFU/mL. At the beginning of the experiment (time zero) and after every 3 h during a 24 h growth period, two samples for each strain were collected and each one underwent serial 10-fold dilutions in duplicate. The growth of each isolate was tested in quadruplicate. A total of 5 µL of the dilutions were drop-plated onto Charcoal Cefoperazone Deoxycholate (16) Agar (CCDA; Oxoid, Ottawa, ON) square plates (VWR, Mississauga, ON) and incubated for 48 h at 37°C under microaerophilic conditions.

2.3.2 Motility assay

Motility assays were done according to the protocol of Golden *et al.* (47) with some modifications. Bacterial cells grown for 24 h on MHA plates were collected and resuspended in MH broth (Figure A1-A, Step A). From cultures diluted to an OD_{600} of 0.025**,** 1 µL of each sample was stabbed into 0.4% agar MH plates (Figure A1-A, Step B) and incubated at 37°C for 48 h under microaerophilic conditions (Figure A1-A, Step C). Using a ruler, the diameter of the circle formed by the movement of

bacterial strains was measured (Figure A1-A, Step D), with the radius corresponding to the displacement of the bacterial strain. The *C. jejuni* FlhA mutant was used as the non-motile negative control.

2.3.3 Biofilm assay

Biofilm formation of *Campylobacter* strains was performed as described by McLennan *et al.* (92) with some modifications. Cultures grown on MHA plates from SSB stock were placed in 4 mL MH broth for 24 h with shaking under microaerophilic conditions (Figure A1-B, Step A). Cultures were then diluted to an OD_{600} of 0.05 in two separate 1.5 mL microfuge tubes for each sample (Figure A1-B, Step B). As biofilm formation was tested at 24, 48 and 72 h, three 96-well microtiter plates were required for each experiment. In all three plates, 100 µL from each sample tube was placed into duplicate wells for a total of four replicates per sample at every time point (Figure A1-B, Step C). Microtiter plates were incubated at 37°C under microaerophilic conditions for 24, 48 or 72 h. After the first 24 h incubation, 25 µL of 1% crystal violet solution in 95% ethanol was added to each well of the 24 h microtiter plate and incubated for 15 min at room temperature (Figure A1-B, Step D). Plates were rinsed thoroughly with water and visually assessed for biofilm formation (Figure A1-B, Step E). To quantify biofilm formation, plates were incubated with 150 µL of DMSO for 24-48 h to dissolve crystal violet before measuring the absorbance at 570nm (Figure A1-B, Step F). Steps D to F were repeated for the 48 and 72 h time points. MH broth was used as a negative control for biofilm formation.

2.3.4 Hydrogen peroxide sensitivity assay

Sensitivity of *Campylobacter* strains to hydrogen peroxide was tested following the disc diffusion method of Ishikawa *et al.* (64) with some modifications. An overnight bacterial culture was diluted to an OD_{600} of 0.2 (approximately $1X10^8$ CFU/mL) (Figure A2, Step A). For each strain, $1x10^7$ CFU/mL was plated onto two MHA plates. Whatman 3MM paper discs (6mm in diameter; Fisher Scientific) with 0.05, 1 and 8 µmoles of hydrogen peroxide were placed onto one plate and 0.1, 2, and 4 µmoles on the second plate (Figure A2, Step B). Plates were incubated at 42°C for 48 h under microaerophilic conditions (Figure A2, Step C). Clear zones surrounding the discs were measured to determine sensitivity of each strain to hydrogen peroxide (Figure A2, Step D).

2.3.5 Adhesion and invasion assays using Caco-2 cells

2.3.5.1 Growth of Caco-2 cells

A frozen stock of the human Caco-2 cell line was obtained from the American Type Culture Collection (ATCC HTB-37, Manassas, VA) at passage 18 and was cultured at 37°C in a 5% CO_2 incubator in a complete growth medium. The Caco-2 cell line is derived from human colon adenocarcinoma (144). They are structurally similar to small intestinal enterocytes and have a comparable differentiation period (144). Following ATCC guidelines, the complete growth medium for Caco-2 cells consisted of 2 mM L-glutamine (Gibco), 1 mM sodium pyruvate (Sigma-Aldrich, Oakville, ON), 1.5 g/L sodium bicarbonate (Sigma-Aldrich), 20% heat inactivated fetal bovine serum (FBS; Invitrogen, Burlington, ON), distilled water (Invitrogen) and Minimal Essential Medium powder mix (Invitrogen) containing both Earl's Balanced

Salt and Non-Essential Amino Acids. Before use, the medium was filtered with a Filtropur V50 filter (Sarsted, Montreal, QC). For the subculturing procedure, 1X PBS (pH 7.2) and 0.25% (w/v) Trypsin-EDTA solution (Invitrogen) were used to split the cells before resuspending in 20% complete growth medium. Culture medium was renewed every 4-5 days until cells reached 80% confluency (approximately 9-10 days). All adhesion and invasion assays were conducted with cells from passages 21 to 25. The Caco-2 cell line has a passage limit of 36 before functional differences are observed (165).

2.3.5.2 Preparation of Caco-2 cells for assays

The adhesion and invasion assay protocol was based on a previous study by Hänel *et al.* (55) with some modifications. Briefly, Caco-2 cell monolayers at 80% confluency were subcultured with 10% complete growth medium (10% FBS) and were subsequently seeded at a concentration of $5x10^5$ cells/well in 24-well tissue culture plates (Fisher Scientific) (Figure A3, Step A1). Two plates were put into a 5% CO_2 humidified atmosphere overnight, one for total bacterial cell counts (Plate 1) and the other for the bacterial cell invasion counts (Plate 2). Prior to the addition of bacterial stocks, cell monolayers were washed once with 1X PBS (pH 7.2).

2.3.5.3 Preparation of bacterial strains for assays

Overnight cultures of *Campylobacter* strains grown microaerobically at 37°C were resuspended in 1X PBS containing 1% FBS and adjusted to an OD_{600} of 0.2 (approximately $1x10^8$ cells) in a final volume of 1 mL (Figure A3, Step A2). For both

Plate 1 and Plate 2, each sample was added in triplicate therefore three tubes diluted to the proper optical density were required for each strain per assay.

2.3.5.4 Adhesion and invasion assays

Campylobacter stocks were added to Caco-2 cell monolayers in 500 µL aliquots (approximately $5x10^7$ cells), and plates were centrifuged for 5 min at 200 x g before incubating 2 h at 37°C in a 5% CO_2 humidified atmosphere to allow bacterial adherence and internalization (Figure A3, Steps B1 and B2). Plate 1 was removed and the cell monolayers were washed three times with 500 µL 1X Earle's Balanced Salt Solution (EBSS; Invitrogen). To recover the total bacterial cell count, cell monolayers were lysed with 500 µL of 1% Triton X-100 for 15 min at room temperature (Figure A3, Step C1). In a 2 mL deep-well plate (Ultident, St. Laurent, QC), serial 10-fold dilutions of bacterial cells were completed (Figure A3, Step D1) before plating 5 µl of each sample on CCDA plates (Figure A3, Step E1). Strains were placed in a microaerophilic environment for 2 days at 37°C to recover. For Plate 2, cell monolayers were washed twice with 500 µL 1X EBSS and incubated in fresh PBS containing 1% FBS and 200 µg/mL of gentamicin for 2 h to kill any remaining viable extracellular bacteria (Figure A3, Step C2). Caco-2 cell monolayers were subsequently washed twice with 500 µL 1X EBSS, lysed with 500 µL 1X Triton X-100 and incubated for 15 min at room temperature to obtain an invasion cell count. A 10-fold serial dilution of samples (Figure A3, Step D2) was plated in 5 µl droplets on CCDA plates and incubated for 2 days at 37°C in a microaerophilic environment (Figure A3, Step E2). Adhesion and invasion assays were conducted in

triplicate per strain over three successive passages. The *C. jejuni* FlhA mutant was used as the non-invasive negative control.

2.4 Microarray experiment

"RNA vs DNA" microarray experiments (Figure A4) were conducted in order to measure the transcript abundance and copy number for each gene as previously described (6, 147, 162). A full genome *Campylobacter* microarray was first used to determine transcript profiles of phage-containing *Campylobacter* isolates and the control strain to assess whether differences in virulence properties were due to variability in gene expression. Next, based on results from phenotypic assays, subsets of 10 strains from each group (clinical, environmental and animal) were chosen for gene-expression analysis. A two-channel microarray experiment was performed with complementary DNA (cDNA) and genomic DNA (gDNA).

2.4.1 RNA extraction protocol

An overnight culture grown on an MHA plate was adjusted to an OD_{600} of 0.2. A 200 µL inoculum was grown for 16-20 h at 37°C under microaerophilic conditions on either MHA plates or MHA supplemented with 10% Laked Horse Blood (Oxoid) plates, for phage-containing isolates. Total RNA was isolated from bacterial cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Briefly, bacterial pellets were resuspended in 5 mL TRIzol solution and cells were homogenized with a 5 mL syringe and an 18-gauge needle or using a Mini Bead-beater (Fisher Scientific) for phage-containing isolates. Lysates were transferred to a 15 mL Polystyrene screw cap tube (Fisher Scientific) before adding 1 mL of

chloroform and incubating for 3 min at room temperature. Samples were centrifuged at 12000 x g for 15 min at 4°C in a Heraeus Multifuge 1 S-R centrifuge (Thermo Scientific) to separate the aqueous phase from the remaining lysate. The addition of 2.5 mL isopropanol to the aqueous phase preceded an incubation at room temperature for 10 min and a 15000 x g spin for 15 minutes at 4°C to recover RNA pellets. A first wash with 5 mL 75% ethanol was performed to remove salts; samples were stored at -20°C for a maximum of 2 weeks. Samples were then centrifuged at 7500 x g for 5 min at 4°C and supernatant was decanted in order to wash the pellet with 5 mL 100% ethanol before centrifuging once more. Using a gel loading pipette tip (VWR), supernatants were removed and pellets resuspended in 100 µL RNase-DNase Free water (Invitrogen). RNA was DNAse treated using Fermentas DNase I (Thermo Scientific). Briefly, a mix of 10 µg of RNA, 5 µL 10x reaction buffer with $MgCl_2$, 5 U of DNase I (Thermo Scientific) and up to 50 µL DNase-RNase Free water (Invitrogen), was incubated for 30 minutes at 37°C. RNA was extracted with 100 µL Trizol reagent (Invitrogen) and 20 µL Chloroform. Following vigorous shaking for 15 seconds and a 3 minutes incubation at room temperature, the sample was centrifuged at 12000 x g for 15 minutes at 4°C. The aqueous phase was transferred to a new tube and RNA was precipitated by adding 84 µL of isopropanol and incubating for 10 minutes prior to centrifuging at 12000 x g for 15 minutes 4°C to recover the pellet. RNA pellet was washed twice with 200 µL 70% ethanol and the pellet was resuspended in RNase-DNase Free water (Invitrogen) to repeat the DNase I treatment. After the second DNase I treatment, the pellet was resuspended in 12 µL RNase-DNase Free water and RNA was quantified using the Nanodrop Spectrophotometer ND-1000 (Fisher Scientific) before storing samples at -80°C. For

a subset of samples, RNA integrity was determined using the 2100 Bioanalyzer (Agilent, Mississauga, ON), with the RNA 6000 Nano Kit (Agilent) according to the manufacturer's instructions.

2.4.2 DNA extraction protocol

Genomic DNA was isolated for the microarray experiment by the method of Carrillo *et al.* (14) with some modifications. A 200 µL inoculum of an overnight culture was adjusted to OD_{600} of 0.2 before incubating for 20-24 h at 37°C in microaerophilic conditions on MHA plates or MHA supplemented with 10% Laked Horse Blood, for phage-containing isolates. Bacterial pellets were resuspended in 2 mL 1X TE buffer (10mM Tris, 10mM EDTA) and cells were homogenized using a 5 mL syringe with 18-gauge needle. After the addition of 25 µL of lysozyme (25 mg/mL; Bioshop), 3 µL of Rnase A (100 mg/mL; Qiagen, Toronto, ON) and 5 µL proteinase K (14 mg/mL; Bioshop, Burlington, ON), a 1 h incubation at 37°C was required. A total of 10 µL of 10% SDS was added and lysates were transferred to MaxTract 15 mL phase-lock tubes (Qiagen). With the addition of an equal volume of phenol: chloroform: isoamyl (Fisher Scientific), sample were centrifuged for 10 min at 1257 x g in Heraeus Multifuge 1 S-R (Thermo Scientific). The addition of the Phenol solution was repeated two more times until the aqueous phase was clear. The aqueous phases were transferred to 15 mL screw cap tubes (Fisher Sceintific) to precipitate the DNA by adding isopropanol and sodium acetate (3M, pH 5.2; Bioshop). After a 20 min incubation, samples were spun at 14957 x g for 15 min at 4°C to recover genomic DNA pellets. Pellet were washed twice with 2 mL 70% ethanol and centrifuged at 14957 x g for 5 min at 4°C. Another wash was done with

1 mL 100% ethanol and pellet was centrifuged at 14957 x g for 5 min at 4°C. Gel loading tips were used to remove supernatant and the DNA pellet was resuspend in 1X TE buffer (10 mM Tris, 1 mM EDTA) followed by a 1 h incubation at 65ºC. DNA was quantified with the Nanodrop Spectrophotometer ND-1000 (Thermo Scientific) before storing sample at -20ºC.

2.4.3 Sonication of genomic DNA

The following protocol was modified from the thesis of S. McIlwham (91). In a 15 mL screw cap tube, gDNA and sonication buffer (50 mM Tris, 10 mM EDTA) were combined for a total volume of 600 µL and the mix was placed on ice. Using the Sonifier 250 (Fisher Scientific), samples were sonicated at a 20% duty cycle and an output control of 2 for 10 s (Figure A5, Step A). Before and after sonication of each sample, the sonifier was washed twice with 2 mL water and once with 2 mL 70% ethanol. To precipitate fragmented DNA (Figure A5, Step B), 1 µL of glycogen, two volumes of ice-cold 95% ethanol, and one-tenth volume of sodium acetate (3 M, pH 5.2; Bioshop) was added and then samples were incubated on ice for 30 min. To recover the DNA, samples were transferred to a 2 mL microfuge tube and centrifuged at 21000 x g for 20 min at 4°C in a Heraeus Multifuge 1 S-R (Thermo Scientific). Pellets were washed twice with 1 mL 70% cold ethanol and then centrifuged at 21000 x g for 5 min at 4°C (Figure A5, Step C). Supernatants were removed and pellets air-dried on ice for 10 min before resuspending in 100 µL of nuclease free water (Figure A5, Step D). Genomic DNA concentration was determined with the Nanodrop Spectrophotometer ND-1000 (Thermo Scientific) and

adequate fragmentation was confirmed by running samples on a 1% agarose gel for 30 min at 110V (Figure A5, Step E). Samples were stored at -20°C.

2.4.4 Labelling of complementary DNA and fragmented genomic DNA

2.4.4.1 From RNA to labelling of complementary DNA

2.4.4.1.1 Reverse transcription of RNA

This method was adapted from the DeRisi protocol (26). For complementary DNA synthesis, 15 µg of total RNA was first adjusted to 15 µL with RNase-free water (Invitrogen). Then 3 µL of Random primers (5 µg/µL; Integrated DNA Technologies, Coralville, IA), 4 µL of dNTP mix (5.0 mM of each dATP, dGTP, and dCTP; Invitrogen), 1 µL of dTTP (10 mM; Invitrogen), 2 µL of amino-allyl dUTP (5.0 mM; Sigma-Aldrich), 8 µL of 5X First Strand Buffer (Invitrogen), 4 µL of DTT (100 mM; Invitrogen), 1 µL of RNase Inhibitor (40 U/µL; Promega, Madison, WI) and 2 µL of Superscript II (Invitrogen) were added for a final reaction volume of 25 µL. The PCR program consisted of a 42°C incubation for 15 min, then a 2 h incubation at 45°C and finally a 5 min 95°C denaturing step using the Mastercycler gradient PCR machine (Fisher Scientific). RNA was hydrolyzed by adding 8 µL of sodium hydroxide (1 M; Bioshop) and then the sample was incubated for 15 min at 65°C. The reaction was neutralized and pH adjusted by the addition of 8 µL hydrochloric acid (1 M; Bioshop) and 4 µL Tris-HCl (1 M, pH 8.0; Bioshop).

2.4.4.1.2 Clean-up of complementary DNA

To prevent a reaction of the amine groups on Tris with the monofunctional NHS-ester of the cyanine dye, a pre-coupling purification using the Microcon YM-30

filters (Invitrogen) was required to remove the Tris. Briefly, 450 µL of water was added to the sample before putting the mix in a Microcon YM-30 column (Invitrogen). The columns were centrifuged at 22464 x g for 8 min in a Galaxy 16DH tabletop centrifuge (VWR). Samples were washed three more times with 450 µL water and then concentrated with a final spin at 22464 x g for 5 min. To recover cDNA, 5 µL of water was added to the columns, the samples were vortexed for 10 s and then the columns were inverted before centrifugation at 22464 x g for 1 min. Complementary DNA was quantified using the Nanodrop Spectrophotometer ND-1000 (Thermo Scientific).

2.4.4.1.3 Labelling of complementary DNA

For the dye coupling reaction, Cy-3 and Cy-5 NHS esters (CyDye; GE Amersham Biosciences, Piscataway Township, NJ) were resuspended in 40 µL Atlas Glass approved DMSO (Clontech, Mountain View, CA). To the 5 µL cDNA samples, 3 µL of Sodium Bicarbonate (300 mM, pH 9; Bioshop) and 2.5 µL of Cy-3 or Cy-5 NHS esters were added before incubating in the dark at room temperature for 1 h. Samples were purified with Qiaquick PCR purification columns (Qiagen), a procedure that first consists of adding 500 µL of PB buffer and centrifuging at 22464 x g for 1 min in VWR Galaxy 16DH tabletop centrifuge. Columns were then washed twice with 600 µL PE buffer. After the second spin at 22464 x g, flow-through was discarded and columns were centrifuged at 22464 x g for 2 min to drive out residual wash buffer. To labelled cDNA, 55 µL water was added and sample was incubated for 5 min at 55°C and then eluted by spinning at 22464 x g for 1 min. Labelling of

complementary DNA was confirmed by testing the absorbance of the Cy dyes with the Nanodrop spectrophotometer ND-1000 (Thermo Scientific).

2.4.4.2 Labelling of sonicated genomic DNA

For labelling of gDNA, Mirus Label IT Cy-3 and Cy-5 dyes kit (Cedarlane, Burlington, ON) were first dissolved in 100 µL resuspension buffer and stored at -20°C. The coupling reaction consisted of 10 µg fragmented DNA, 5 µL of 10X Label IT buffer A and 2.5 µL of Cy-dye in a final volume of 50 µL. The reaction mix was incubated in the dark at 37°C for 18 h prior to a first purification with SigmaSpin Colum Clean-Up (Sigma-Aldrich) according to manufacturer's instructions. A second purification was performed with Qiaquick PCR purification column (Qiagen) as previously described for dye-coupled complementary DNA.

Purified complementary and genomic DNA probes were centrifuged for 40 min at 45°C down to near dryness in the Vacufuge Plus (Fisher Scientific). When necessary, the samples were stored in the dark at -20°C.

2.4.5 Array hybridization procedure

2.4.5.1 Denaturation of probes

Before inserting into the GeneTAC Hyb station (Genomic solutions, Ann Arbor, MI), denaturation of complementary and genomic DNA probes was done separately. For complementary DNA, probes were denatured by heating at 100°C for 5 min and a subsequent incubation for 5 min on ice. For genomic DNA, probes were denatured according to the Mirus instructions and reagents. Briefly, samples were placed at room temperature for 5 min and then on ice for 1 min before adding

the D1 Buffer. Secondly, N1 Buffer was added and samples were incubated at room temperature for 5 min, heated at 100°C for 5 min and then put on ice for 5 min.

2.4.5.2 Microarray slide and the GeneTAC Hyb station

A pan-genomic *C. jejuni* microarray consisting of PCR products of 1643 genes from NCTC11168 and 494 unique genes from RM1221, 81-176, pTet and pVir was used for the analysis of all *Campylobacter* isolates. On the day of the hybridization experiment, microarray slides were first placed in a blocking solution (5X SSC, 0.1% SDS, 0.1µg/µL BSA, 40% formamide) for 45 min at 45°C in a water bath (VWR). Slides were then washed twice with distilled water and then with isopropanol. Slides were dried by centrifuging at 500 x g for 5 min in a RC5C centrifuge (Mandel Scientific Co. LTD, Guelph, ON) and then were placed in a GeneTAC Hyb Station (Genomic solutions), following manufacturer's protocol.

Genomic DNA and complementary DNA probes were combined prior inserting into the Hyb Station for an 18 h run. When the hybridization procedure was complete, slides were removed from the Hyb Station and dried for 5 min at 500 x g. Microarrays were scanned in the VersArray Chip Reader 5 µm system (Bio-Rad, Mississauga, ON) and data was analyzed using Array-Pro Analyzer (Media Cybernetics, Rockville, MD).

2.4.6 Analysis of microarray data

Results for phage-containing isolates and the control strain were analyzed with the Array-Pro Analyzer (Media Cybernetics). By combining a high and a low photomultiplier tube (PMT) scan with GeneMaths XT (Applied Maths, Austin, TX), a

greater dynamic range of the cDNA channel was obtained. In order to measure the relative quantity of RNA, the intensity of each spot in the cDNA channel was divided by the intensity of the respective spot in the DNA channel. Per chip normalizations were performed using the 50^{th} percentile of all measurements in each sample. Each gene was normalized to itself by dividing the value of that gene by the median of its measurements in all samples. Statistically significant differences among strains (00-2425, 00-2426, 00-2538, 00-2544) were determined using the non-parametric Kruskal-Wallis test (110).

For microarrays of clinical, animal and environmental strains, analysis was limited to determining the presence of a signal in each channel (RNA or DNA). The intensity values of replicate gene spots on arrays were mean-normalized. A ratio of raw intensity and background above 2 was considered expressed (RNA channel) or present (DNA channel). Strains from each source (clinical, animal and water) were grouped together to determine the number of strains in each source that expressed or contained each gene. Genes that were not present or expressed in at least one strain were removed from the analysis. The percentage of strains carrying (or expressing) a gene in each source was determined by dividing the number of strains having the gene with the total number of strains analysed in the respective source for each channel (RNA or DNA) separately. The presence (or expression) of a gene in each source was compared using the Fisher exact test to find statistically significant differences ($p<0.05$) (146).

2.5 Real-time PCR of *C. jejuni* phage-containing isolates

Following the microarray analysis, Sybr Green (Quanta Biosciences, Gaithersburg, MD) Real-Time PCR experiments with the MX3005P QPCR System (Agilent) were done for a number of genes found to be up-regulated in *C. jejuni* phage-containing isolates (00-2425, 00-2538 and 00-2544) relative to the control strain (00-2426), were performed using cDNA of the respective strain and gene-specific primers (Table A2). Three housekeeping genes, *AspA*, *GltA* and *GlyA*, were run as controls on each plate.

After an initial denaturation step of 95°C for 10 seconds, PCR amplification consisted of 40 cycles with 95°C for 15 seconds and 53°C for 60 seconds. Samples were maintained at 4°C when the run was complete. Relative gene expression was calculated using the delta delta Ct method (13).

2.6 Knockout mutant construction of the *C. jejuni* NCTC11168 *Cj0075c, Cj0199c, Cj0305c, Cj0809c and Cj1373* genes

C. jejuni NCTC 11168 mutants (Table A3) were constructed by inactivating *Cj0075c*, *Cj0199c*, *Cj0305c*, *Cj0809c* and *Cj1373* genes as described by Palyada *et al.* (113). These genes were selected, as they appeared to be over-represented in strains implicated in clinical strains of infection. They were chosen from a larger pool of strains that have been typed by CGH and that might be implicated in human infection.

Briefly, using gene-specific primers with a 16 bp homology to pUC19 (Table A4) and *Taq* DNA polymerase (Invitrogen), the genes to be mutated were amplified by PCR from the chromosomal DNA of *C. jejuni* NCTC11168. The PCR products

(1,073-bp for Cj0075c; 1,113-bp for Cj0305c; 1,284-bp for Cj0199c; 1,010-bp for Cj0809c; 2,676-bp for Cj1373) were cloned into a *Bam*HI unique restriction site in the pUC19-digested plasmid using the In-Fusion Dry-Down PCR Cloning Kit (Clontech) following manufacturer's instructions. Recombinant plasmids were transformed into *E. coli* DH5α and the insertion of target genes was confirmed by PCR. By doing inverse PCR experiments with gene-specific primers containing a 15 bp homology to cat (Table A4), deletions of 262, 284, 120, 69 and 1677 bp were generated within *Cj0075c*, *Cj0199c*, *Cj0305c*, *Cj0809c* and *Cj1373*, respectively. The chloramphenicol resistance cassette, PCR amplified from pRY111 plasmid, was cloned into the inverse PCR products resulting in plasmids pGA0075c, pGA0199c, pGA0305c, pGA0809c, pGA1373. Due to time constraints, only two plasmids were successfully naturally transformed into *C. jejuni* NCTC 11168, yielding Δ*Cj0075c* and Δ*Cj1373*. The presence of a disrupted gene was confirmed by growing strains on plates supplemented with 200 μg/mL chloramphenicol, by PCR and also by sequencing with gene-specific primers.

3: RESULTS

3.1 Impact of phage on virulence of *C. jejuni*

In a 2012 study, Clark *et al.* (19) showed that phage-containing *Campylobacter* isolates were more virulent than those lacking phage, as indicated by an increase in adherence and invasion for the phage-containing isolates. To assess the impact of phage on the virulence of *C. jejuni*, three phage-containing *C. jejuni* clinical isolates with sequence homologs to the temperate *Campylobacter* Mu-Like Phage 1 (CMLP1) found in the chicken isolate RM1221 were analysed. A genetically related clinical strain lacking the phage insertion was used as a control. All of these strains are from the same subtype and have the same gene content when compared by microarray experiments except for the absence of the CJIE1 prophage in the control strain 00-2426 (19). When comparing PFGE patterns of the four strains, genome sizes were calculated and strain 00-2426 had 39 kb less due to the absence of CJIE1 (19). Also, Southern blotting experiments using RM1221 CJIE1 prophage genes showed that these genes were probe-positive in phage-containing isolates (00-2425, 00-2538, 00-2544) but not the control strain (00-2426) (20). Previous growth curve experiments determined that these four strains have similar growth rates.

3.1.1 Phenotypic assays

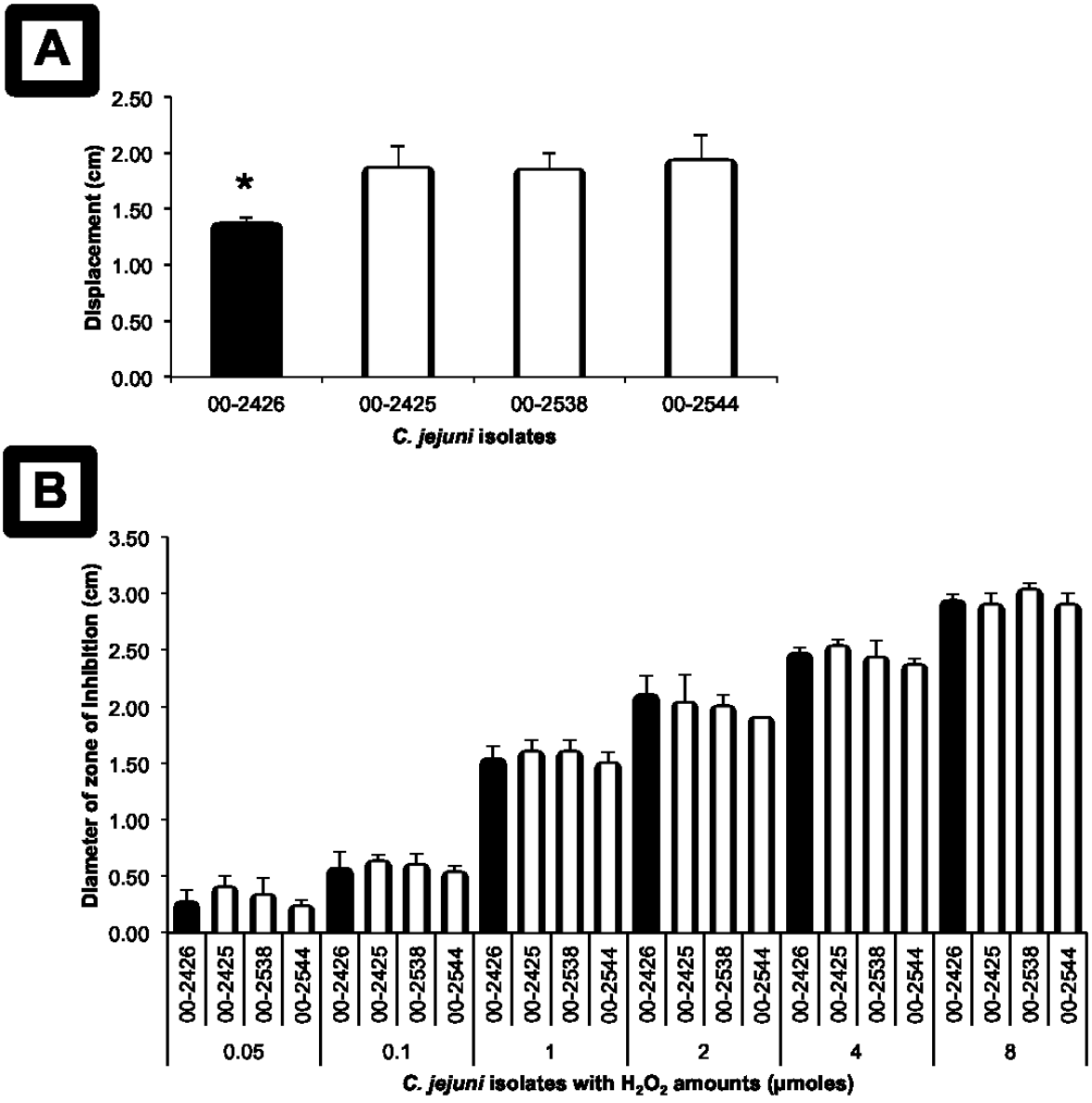
3.1.1.1 Impact of phage on *C. jejuni* motility

To determine if the insertion of phage into the *C. jejuni* genome influences bacterial motility, assays were performed on 4 *C. jejuni* strains from the Walkerton *E. coli* and *Campylobacter* outbreak. Three strains, 00-2425, 00-2538 and 00-2544, containing a *Campylobacter* Mu-like prophage (CMLP1) were compared with the control strain, 00-2426, devoid of prophage (Figure 2A).

The motility of each strain was assessed by measuring the radius of the circle produced by bacterial movement when stabbed in the middle of a soft agar plate. The radius, representing the displacement, of each strain was determined in three replicate experiments and the mean displacement was calculated.

The mean displacement of the control strain 00-2426 was found to be significantly different than that of the phage-containing isolates 00-2425 (Student T-test, $p=0.006$), 00-2538 ($p=0.003$) and 00-2544 ($p=0.007$). Furthermore, the mean displacement of the three phage-containing isolates was significantly higher than that of the control strain ($p=0.004$). The presence of phage in these isolates leads to more motile strains. However, with only four strains being tested, the significance of this observation cannot be determined without first testing a greater number of strains. Unfortunately, genetically similar strains with and without prophage, have not yet been identified.

Figure 2. Motility and sensitivity to hydrogen peroxide of phage-containing *C. jejuni* isolates. For the motility assay (Figure 2A), a 1 µl suspension of each strain was stabbed into 0.4% MH agar plates and incubated for 48 h under microaerophilic conditions. Motility was determined by measuring the radius of the circle created by bacterial movement. Error bars represented standard deviation of displacement. The control strain 00-2426 (Black bars) was significantly (*) less motile than the phage-containing *C. jejuni* isolates (White bars) with a p-value of 0.004. For the sensitivity assay (Figure 2B), bacterial lawns of three phage-containing *C. jejuni* isolates (White bars) and the control strain (Black bars) were challenged with 0.05, 0.1, 1, 2, 4 and 8 µmoles of hydrogen peroxide. Larger zone of inhibition indicated lower resistance to hydrogen peroxide. Experiments were repeated three times for all four strains and error bars represented the standard deviation. No significant differences were observed between the phage-containing isolates and the control strain.


A
Displacement (cm)
2.50
2.00
1.50
1.00
0.50
0.00
*
00-2426
00-2425
00-2538
00-2544
C. jejuni isolates
B
Diameter of zone of Inhibition (cm)
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
00-2426
00-2425
00-2538
00-2544
0.05
0.1
1
2
4
8
C. jejuni isolates with H2O2 amounts (µmoles)

3.1.1.2 The influence of phage on sensitivity of *C. jejuni* to hydrogen peroxide

Oxidative stress response of pathogenic bacteria has been widely studied, as it is important for bacterial survival inside the human host and the environment. The hydrogen peroxide assay provides a measure of the sensitivity of bacterial strains to the presence of this oxidant.

To determine if hydrogen peroxide affects phage-containing *C. jejuni* isolates differently than the strains without phage, sensitivity assays were conducted. Disks containing increasing concentrations of hydrogen peroxide were placed on bacterial lawns to determine tolerance of strains to this compound. Mean diameters of zones of inhibition for three biological replicates of each strain were plotted (Figure 2B), and demonstrated increased sensitivity with higher amounts of hydrogen peroxide for the four strains. No significant differences were observed between strain 00-2426 and the combined values of three phage-containing isolates for the varying amounts of hydrogen peroxide.

3.1.1.3 The importance of phage for *C. jejuni* biofilm formation

Bacterial biofilms are commonly found inside patients and in the environment, aiding in survival of the bacteria (85, 151). To determine the influence of prophage on biofilm formation in *Campylobacter* strains, phage-containing *C. jejuni* isolates (00-2425, 00-2538 and 00-2544) and a strain devoid of phage (00-2426) from the Walkerton *E. coli* and *Campylobacter* outbreak, were tested for their ability to form biofilms.

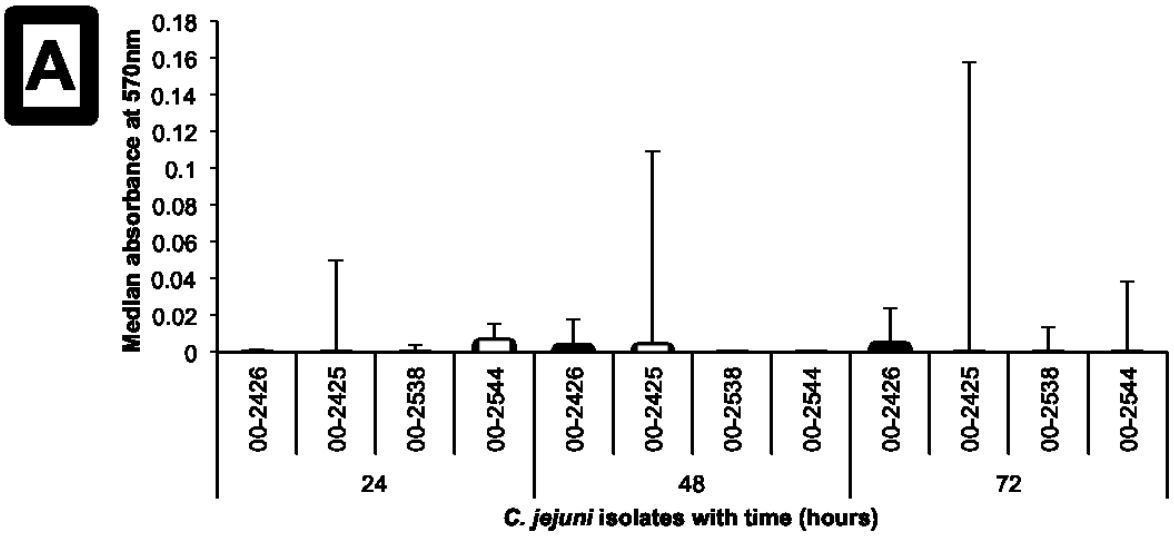
Strains were analysed for biofilm formation with four replicates in each of the four individual experiments performed on different days. For every experiment, a

median absorbance for each isolate was obtained by normalizing the measurements of the 4 replicates of the strain to the 4 replicates of the control (MH broth) in the same plate. Median absorbance measurements of each strain were compared with the control using a Student's T-test. Strains with statistically different median absorbance measurements relative to the control were given a score of 1 for biofilm formation, while other isolates were given a score of 0 for no biofilm formation. This analysis was performed for each of the 4 individual experiments. Results for median absorbance, mean absorbance and biofilm formation ability were calculated from the median of each of the 4 replicate experiments (Figure 3).

There were no observed differences between 00-2426 and the three phage-containing *C. jejuni* isolates in their ability to form biofilms at 24, 48 and 72 h (Figure 3). Data points where the median was zero but the standard deviation was not, such as that of strains 00-2425, 00-2426 and 00-2538 after 24 h (Figure 3A), could have been explained by the wide distribution of data after combining results from the different experiments. Data obtained for strains 00-2538 and 00-2544 after 48 h incubation also showed a median and standard deviation of zero. In the latter case, all results from the 4 experiments illustrated no biofilm formation and therefore the calculated absorbance and standard deviation were zero.

Biofilm formation among strains was further compared by determining differences in mean absorbance measurements (Figure 3B). No significant differences in biofilm formation between the control strain and the three phage containing isolates were observed at 24 (Student T-test, $p=0.3$), 48 ($p=0.4$) and 72 h ($p=0.3$). The influence of outliers on the average absorbance calculated is evident

Figure 3. Biofilm formation of *C. jejuni* phage-containing isolates and the control strain. Biofilm formation was determined for bacterial cultures grown for 24, 48 and 72 h by staining with crystal violet. Plots of the median (Figure 3A) and the mean (Figure 3B) absorbance measurements of 4 replicates for each strain per plate were calculated. Errors bars represented standard deviation. The presence or absence of biofilm formation was also determined (Figure 3C). No significant differences were observed between phage-containing *C. jejuni* isolates (White bars) and the control strain (Black bars).


A
Median absorbance at 570nm
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
00-2426
00-2425
00-2538
00-2544
00-2426
00-2425
00-2538
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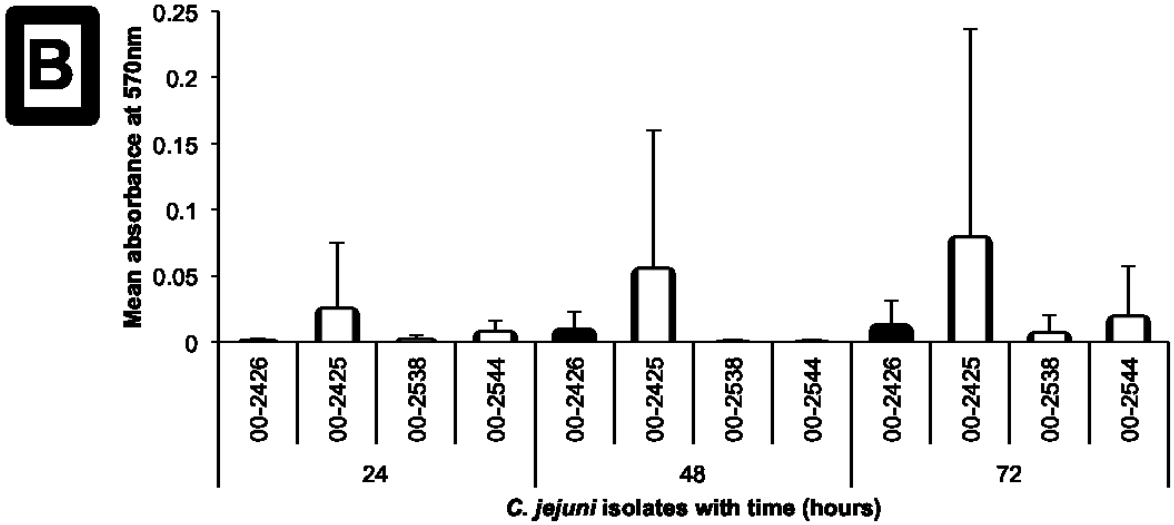
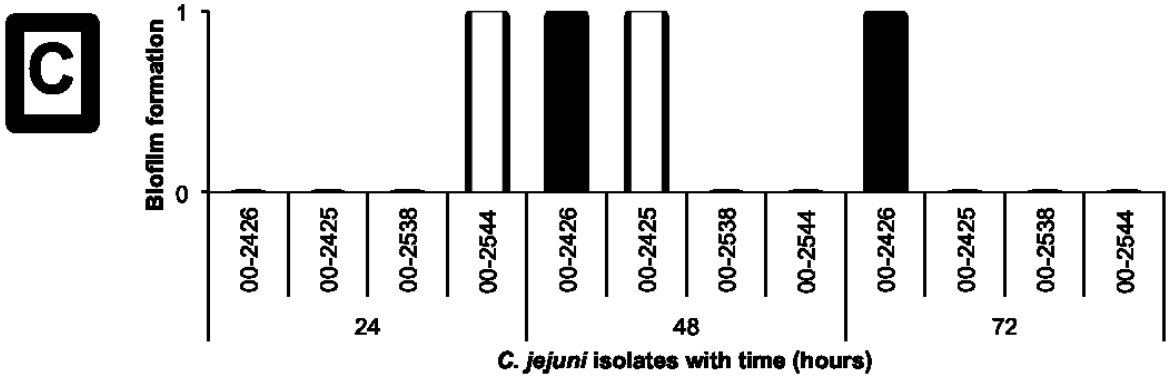
00-2426
00-2425
00-2538
00-2544
24
48
72
C. jejuni isolates with time (hours)


B
Mean absorbance at 570nm
0.25
0.2
0.15
0.1
0.05
0
00-2426
00-2425
00-2538
00-2544
00-2426
00-2425
00-2538
00-2544
00-2426
00-2425
00-2538
00-2544
24
48
72
C. jejuni isolates with time (hours)


C
Biofilm formation
1
0
00-2426
00-2425
00-2538
00-2544
00-2426
00-2425
00-2538
00-2544
00-2426
00-2425
00-2538
00-2544
24
48
72
C. jejuni isolates with time (hours)

(Figures 3A and 3B), indicating the importance of taking the median for a more accurate representation of the data distribution. For example, strain 00-2425 appeared to have the highest mean absorbance measurements at 24, 48 and 72 h. However, when looking at statistically significant biofilm formers (Figure 3C), strain 00-2425 scored zero for the 24 and 72 h time points. The control strain 00-2426 produced biofilms after longer incubation times of 48 and 72 h when compared to the phage-containing isolates 00-2544 and 00-2425 that both formed biofilms after 24 and 48 h, respectively. All strains displayed similar biofilm-forming potential however, it was difficult to make any definitive conclusions without testing a larger number of strains with and without *Campylobacter* phage.

3.1.2 Transcript profiles

To determine if the expression of virulence genes was increased in the presence of prophage, transcript profiles were generated by whole-genome microarray analyses. Using a whole genome *C. jejuni* NCTC11168 "RNA vs DNA" microarray experiment, phage-containing isolates and the control strain were grown to late log phase to compare the relative expression of genes. Labelled complementary DNA and genomic DNA for each sample were hybridized on a microarray slide, the data was normalized and statistical analysis was performed using the Kruskal-Wallis test (110). To measure the relative quantity of RNA, the intensity of each spot in the cDNA channel was divided by the intensity of the respective spot in the DNA channel.

When comparing higher transcript levels in all three phage-containing isolates (00-2425, 00-2538, 00-2544) relative to the control strain (00-2426), 35 genes had

significantly higher transcript levels in all three phage-containing isolates (Table 1). This list was populated using the Array-Pro Analyzer (Media Cybernetics) software t select genes that were two times more highly expressed in all three phage-containing isolates relative to the control strain. These genes identified are involved in several metabolic activities and pathways.

3.1.3 Real-time PCR analysis

To confirm microarray results, genes *Cj0013*, *Cj0035*, *Cj0177*, *Cj0178*, *Cj0311*, *Cj0332c*, *Cj0333c*, *Cj0342c*, *Cj0345*, *Cj0606*, *Cj0951c*, *Cj1392*, *Cj1638* and *Cj1663* were further analyzed using real-time PCR. These genes were chosen based on their putative metabolic activities in *C. jejuni* (116). Transcript levels in the control strain (00-2426) were compared with those in the three phage-containing isolates (00-2425, 00-2538, 00-2544). Three housekeeping genes *GlyA, GltA* and *AspA* were used as internal controls. The delta delta Ct (ΔΔCt) was calculated to determine the relative expression of each gene. This analysis revealed no statistically significant differences by Anova analysis between the phage-containing isolates and the control strain for all genes tested.

3.2 Clinical, animal and environmental *Campylobacter* isolates

The virulence of *Campylobacter* is dependent not only on the presence of genetic traits, but on expression of these traits as strain phenotypes. Clinical, animal and water isolates of *Campylobacter* were compared, to ultimately identify traits that may influence survival and pathogenicity of these strains in these different environments.

Table 1. Genes overexpressed in invasive clinical phage-containing *C. jejuni* isolates relative to the less invasive control isolate. Two channel microarrays of complementary and genomic DNA of each strain were compared to determine the relative copy number of RNA. Genes that were greater than 2x more highly expressed in all three phage-containing isolates (00-2425, 00-2538, 00-2544) relative to the control strain (00-2426) were identified. Statistical analysis was done using the Kruskal-Wallis test. Up-regulated genes were found to be involved in metabolic activities such as amino acid biosynthesis, degradation of macromolecules, ribosomal protein synthesis, DNA modifications, and energy metabolism, protein modification and synthesis. Annotations of genes were obtained from NCBI for strain NCTC11168 (Taxonomy ID: 192222).

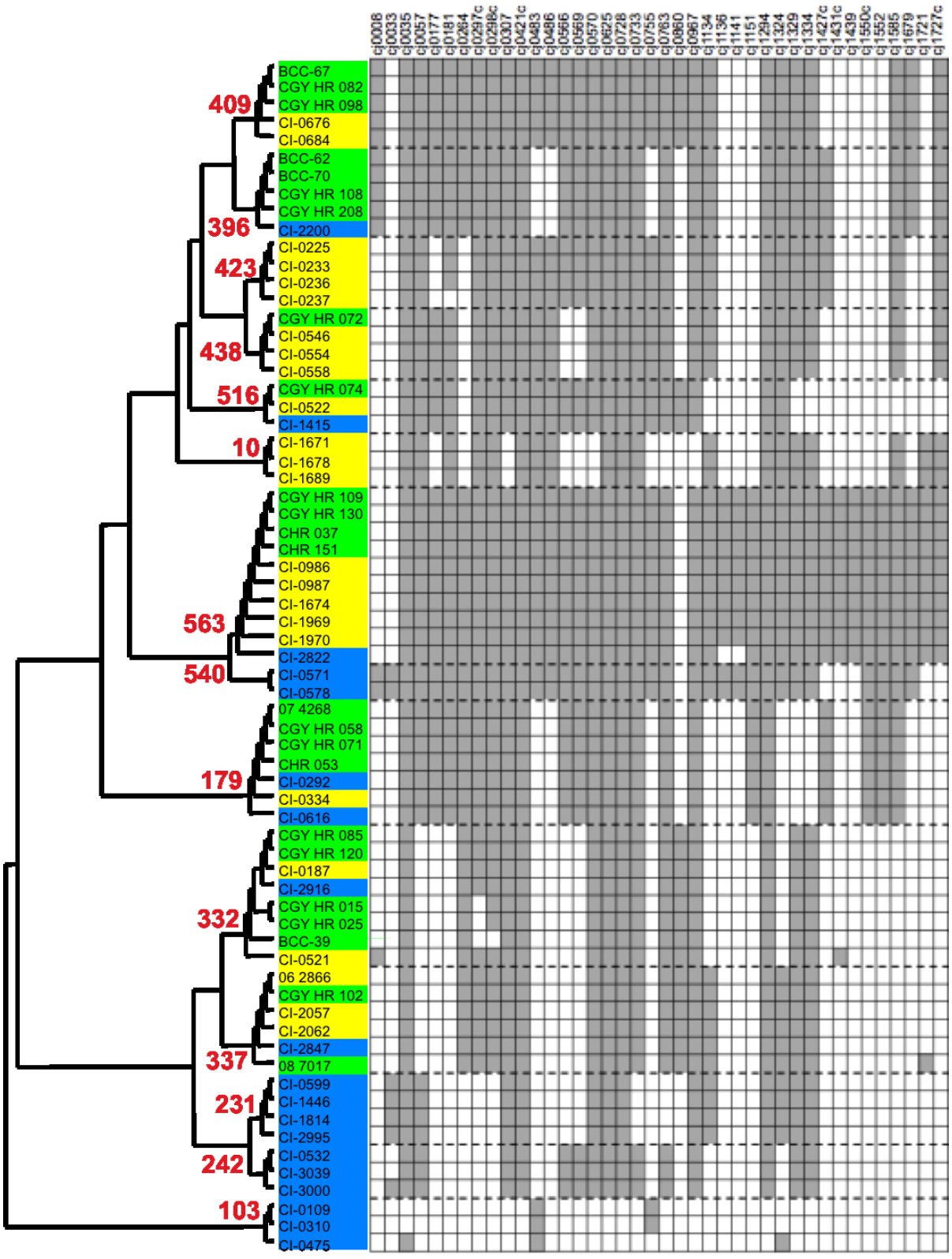
Gene	Description
IlvD (Cj0013)	Dihydroxy-Acid Dehydratase
Cj0035c	Putative Efflux Protein
SlyD (Cj0115)	FKBP-Type Peptidyl-Prolyl Cis-Trans Isomerase
Cj0177	Putative Iron Transport Protein
Cj0178	Putative TonB-Dependent Outer Membrane Receptor
RpmI (Cj0244)	50s Ribosomal Protein L35
RplT (Cj0245)	50S Ribosomal Protein L20
Cj0311	50S Ribosomal Protein L25/General Stress Protein Ctc
Ndk (Cj0332c)	Nucleoside Diphosphate Kinase
FdxA (Cj0333c)	Ferredoxin
UvrA (Cj0342c)	Excinuclease ABC Subunit A
TrpE (Cj0345)	Putative Anthranilate Synthase Component I
RpmB (Cj0450c)	50S Ribosomal Protein L28
RplA (Cj0475)	50S Ribosomal Protein L1
RpsG (Cj0492)	30S Ribosomal Protein S7
QueA (Cj0577c)	S-Adenosylmethionine:tRNA Ribosyltransferase-Isomerase
Cj0606	Putative Secretion Protein HlyD
PstS (Cj0613)	Putative Periplasmic Phosphate Binding Protein
MurF (Cj0795c)	UDP-N-Acetylmuramoyl-Tripeptide D-Alanyl-D-Alanine Ligase
Cj0919c	Putative ABC-Type Amino-Acid Transporter Permease Protein
Cj0951c	Putative MCP-Domain Signal Transduction Protein
Ssb (Cj1071)	Single-Stranded DNA Binding Protein
RpsR (Cj1072)	30S Ribosomal Protein S18
Cj1099	Peptidase (M3 Family)
Tsf (Cj1181c)	Elongation Factor TS
AtpB (Cj1204c)	F0F1 ATP Synthase Subunit A
MetC' (Cj1392)	Putative Cystathionine Beta-Lyase, N-Terminus
RpmJ (Cj1591)	50S Ribosomal Protein L36
RplQ (Cj1596)	50S Ribosomal Protein L17
DnaG (Cj1638)	DNA Primase
Cj1663	Putative ABC Transport System ATP-Binding Protein
RplR (Cj1691c)	50S Ribosomal Protein L18
RplN (Cj1697c)	50S Ribosomal Protein L14
RpmC (Cj1699c)	50S Ribosomal Protein L29
RplP (Cj1700c)	50S Ribosomal Protein L16

3.2.1 Strain selection

A total of 67 *Campylobacter* strains were selected from a pool of human, animal and environmental isolates that had been genetically characterized by comparative genomic fingerprinting (CGF). This multiplex-PCR based assay determines the presence or absence of 40 highly variable genes allowing for the identification of genetically related strains. Based on CGF typing data, a phylogenetic tree was created to infer genetic relatedness of strains (Figure 4). The strains selected for this study were representative of larger pools of isolates in each of the clusters. Cluster composition in the larger dataset (approximately 14 000 strains) was used to determine if strains in a cluster were known to be associated with clinical cases or restricted to water and/or environmental sources (Table B1). Clusters chosen were commonly identified genotypes, mainly selected from the Alberta region to ensure that differences observed were not due to geographic variability.

Although water clusters 232 and 242 were selected from a larger pool that included strains from other sources, in the phylogenetic tree (Figure 4) water strains were grouped together, while clinical and animal isolates were genetically related to each other. For the analysis of the 67 strains, isolates were compared by clusters and by sources. Of these strains, clusters consisting mainly of animal isolates included 10, 423 and 438 (N=11) while water clusters were 103, 231, 242 and 540 (N=12). Clusters containing clinical and animal isolates included 409 and 563 (N=15). When compared by sources, isolates were grouped as clinical (N=24), animal (N=24) or water (N=19) strains.

Figure 4. Genetic relatedness of *Campylobacter* strains from clinical, animal and environmental sources. Sixty-seven *Campylobacter* isolates from clinical (Green), animal (Yellow) and water (Blue) sources were typed using Comparative Genomic Fingerprinting (CGF) analysis (On the right). Using CGF experiments, the presence (Gray square) or absence (White square) of 40 highly variable genes (On the top right) led to the categorization of isolates with distinct genetic fingerprints, also called clusters (Dashed lines). Results from CGF experiments (On the right) were used to create a phylogenetic tree (On the left), illustrating genetic relatedness of all isolates in their respective clusters (Red).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cj0566
cj0569
cj0570
cj0625
cj0728
cj0733
cj0755
cj0763
cj0860
cj0967
cj1134
cj1136
cj1141
cj1151
cj1294
cj1324
cj1329
cj1334
cj1427c
cj1431c
cj1439
cj1550c
cj1552
cj1585
cj1679
cj1721
cj1727c
BCC-67
CGY HR 082
CGY HR 098
CI-0676
CI-0684
BCC-62
BCC-70
CGY HR 108
CGY HR 208
CI-2200
CI-0225
CI-0233
CI-0236
CI-0237
CGY HR 072
CI-0546
CI-0554
CI-0558
CGY HR 074
CI-0522
CI-1415
CI-1671
CI-1678
CI-1689
CGY HR 109
CGY HR 130
CHR 037
CHR 151
CI-0986
CI-0987
CI-1674
CI-1969
CI-1970
CI-2822
CI-0571
CI-0578
07 4268
CGY HR 058
CGY HR 071
CHR 053
CI-0292
CI-0334
CI-0616
CGY HR 085
CGY HR 120
CI-0187
CI-2916
CGY HR 015
CGY HR 025
BCC-39
CI-0521
06 2866
CGY HR 102
CI-2057
CI-2062
CI-2847
08 7017
CI-0599
CI-1446
CI-1814
CI-2995
CI-0532
CI-3039
CI-3000
CI-0109
CI-0310
CI-0475
409
396
423
438
516
10
563
540
179
332
337
231
242
103

3.2.2 Phenotypic analysis

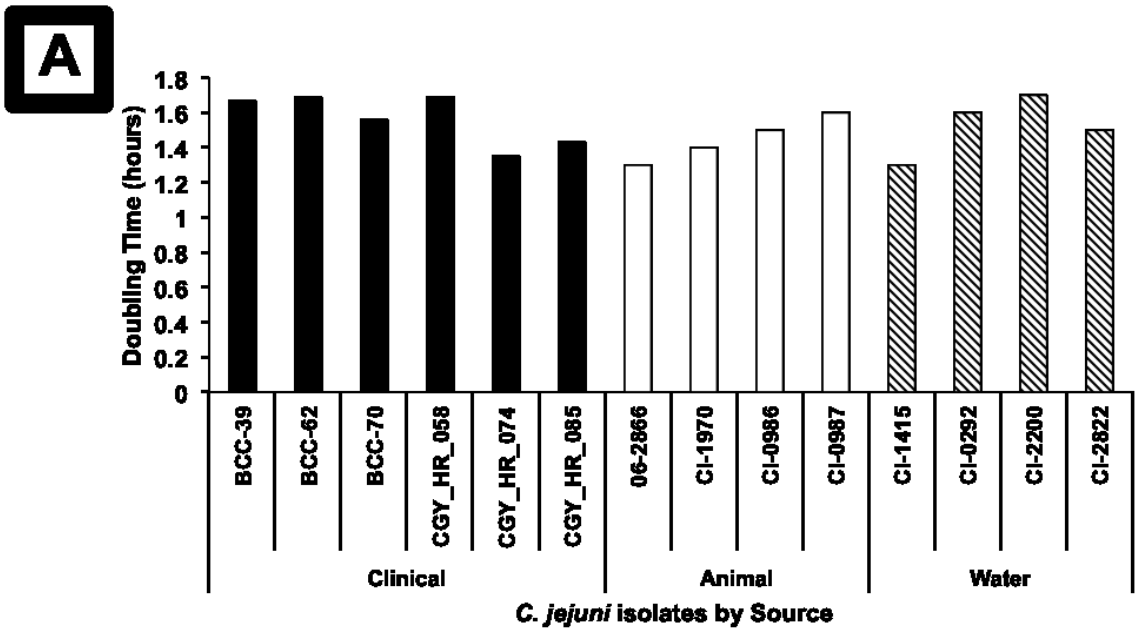
Variability in the phenotypic characteristics of *Campylobacter* spp. isolated from different sources was assessed for motility, adherence, invasion, biofilm formation and resistance to hydrogen peroxide.

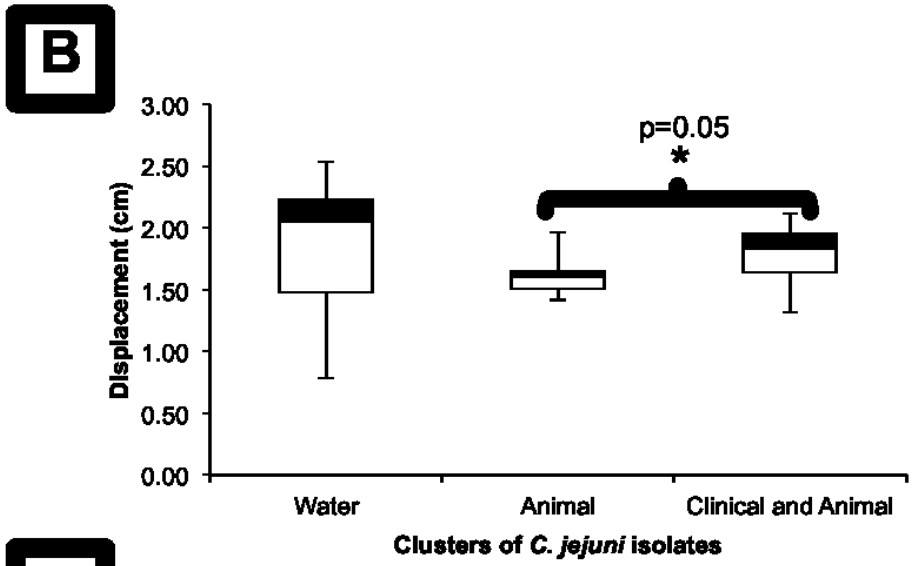
3.2.2.1 Similar growth rates of *Campylobacter* isolates

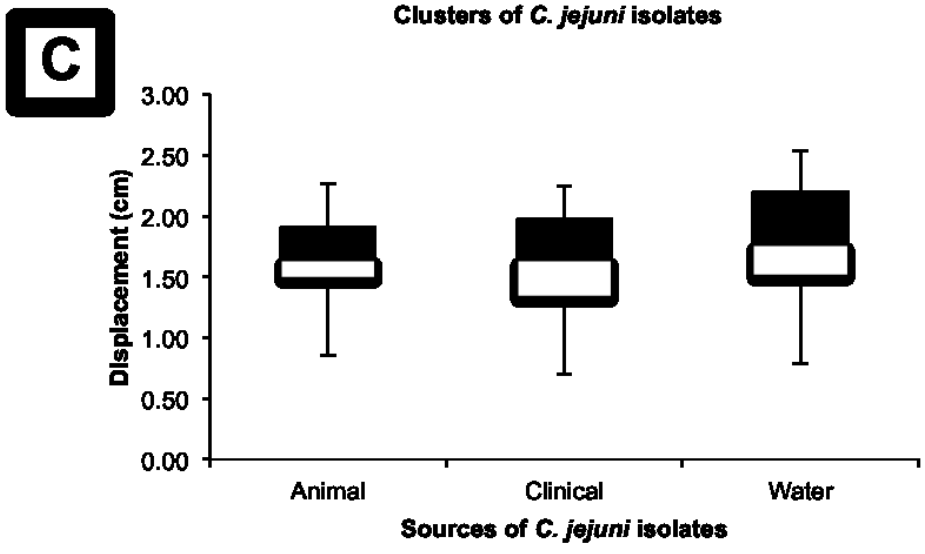
Despite the sensitivity of *Campylobacter* spp. to unfavourable conditions, these bacteria are very widespread, and can be found in several hosts as well as the environment (92, 131, 155). To determine whether the original source of isolation influences the growth rates of *Campylobacter* isolates, experiments were conducted in the same growth medium and incubation conditions for all strains (Figure 5A). Fourteen (6 from clinical, 4 from animal and 4 from water sources) *Campylobacter* strains from clinical, animal and environmental sources were randomly selected from the three source groups.

A growth curve was done for each strain with 4 replicates plated every 3 h for a total period of 24 h. The mean value of the 4 replicates at each time point was used to plot the growth curve. From this growth curve, the doubling time for each strain was calculated using the equation $N(t) = C(2)^{t/d}$, where "N(t)" was the number of bacteria at time point "t", "C" was the initial number of bacteria at time zero and "d" was the time it took the bacteria to double. At 37°C, strains from different sources had similar doubling times ranging from 1.3 to 1.7 h. A Student T-test statistical analysis revealed no significant differences between strains from clinical and animal ($p=0.1$), clinical and water ($p=0.4$) and animal and water ($p=0.3$) sources.

Figure 5. Doubling times and motility for *Campylobacter* strains from clinical, animal and water sources. For growth curves (Figure 5A), a 24 h growth curve was plotted for each sample by plating serial dilutions on MH agar plates every 3 h to determine viable cell counts. Doubling times were calculated from exponential growth curves for 6 clinical (Black), 4 animal (White), and 4 water (Cross-hatched) isolates to assess growth rates of *Campylobacter* strains from different source groups. No significant differences were observed between source groups. Values for doubling times ranged between 1.3-1.7 h for clinical, animal and water sources. For the motility assay, 67 strains from varied sources were placed in the center of 0.4% MH agar plates and incubated for 48 h. The displacement was the radius of the circle produced by the movement of the motile bacteria in the plate. Results were presented in box plots. For each group of strains, the box, which captures the interquartile range is split by the median line and is flanked by vertical lines representing the interval between the lowest (bottom) and the highest (top) values. The isolates were separated into clusters of water (N=12), animal (N=11) as well as animal and clinical (N=15) strains (Figure 5B). *Campylobacter* isolates present in animal clusters were significantly less motile than strains in clusters of animal and clinical isolates combined (p=0.05). Box plots of displacements were created for strains according to their source (Figure 5C). Clinical (N=24), animal (N=24) and water (N=19) *Campylobacter* strains had similar motility when analyzed according to their source.


A
Doubling Time (hours)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
BCC-39
BCC-62
BCC-70
CGY_HR_058
CGY_HR_074
CGY_HR_085
06-2866
CI-1970
CI-0986
CI-0987
CI-1415
CI-0292
CI-2200
CI-2822
Clinical
Animal
Water
C. jejuni isolates by Source


B
Displacement (cm)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
p=0.05
*
Water
Animal
Clinical and Animal
Clusters of C. jejuni isolates


C
Displacement (cm)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Animal
Clinical
Water
Sources of C. jejuni isolates

3.2.2.2 Motility of *Campylobacter* isolates from clinical, animal and water sources

Motility, an important virulence factor in *Campylobacter*, plays a role in adhesion, invasion, chemotaxis, agglutination and biofilm formation. To determine the influence of strain source on motility, assays were conducted to compare clinical, animal and water *Campylobacter* isolates. Strains were separated according to their clusters and sources.

The mean displacement of *Campylobacter* isolates varied according to their clusters. Isolates in clusters containing mostly animal strains (Clusters 438, 10 and 423; N=11), water isolates (Clusters 231, 103, 242 and 540; N=12) or animal and clinical strains (Clusters 563 and 409; N=15) were compared (Figure 5B). Clusters of water isolates had a wider range of motility compared to other clusters. Strains in the water clusters appeared to be more motile than isolates in mostly animal clusters and clusters containing animal and clinical strains, but these differences were not statistically significant. A significant difference in motility was observed when comparing strains in animal clusters to isolates in animal and clinical clusters (Mann-Whitney test; p=0.05). The distribution of data in animal clusters was very small, indicating similar motility for the majority of isolates.

Campylobacter strains were compared for motility by the sources of isolation that included clinical (N=24), water (N=19) and animal (N=24) (Figure 5C). Statistical analysis indicated no significant differences between isolates from animal and clinical (Mann-Whitney test; p=0.4), animal and water (p=0.2), or clinical and water (p=0.1) sources.

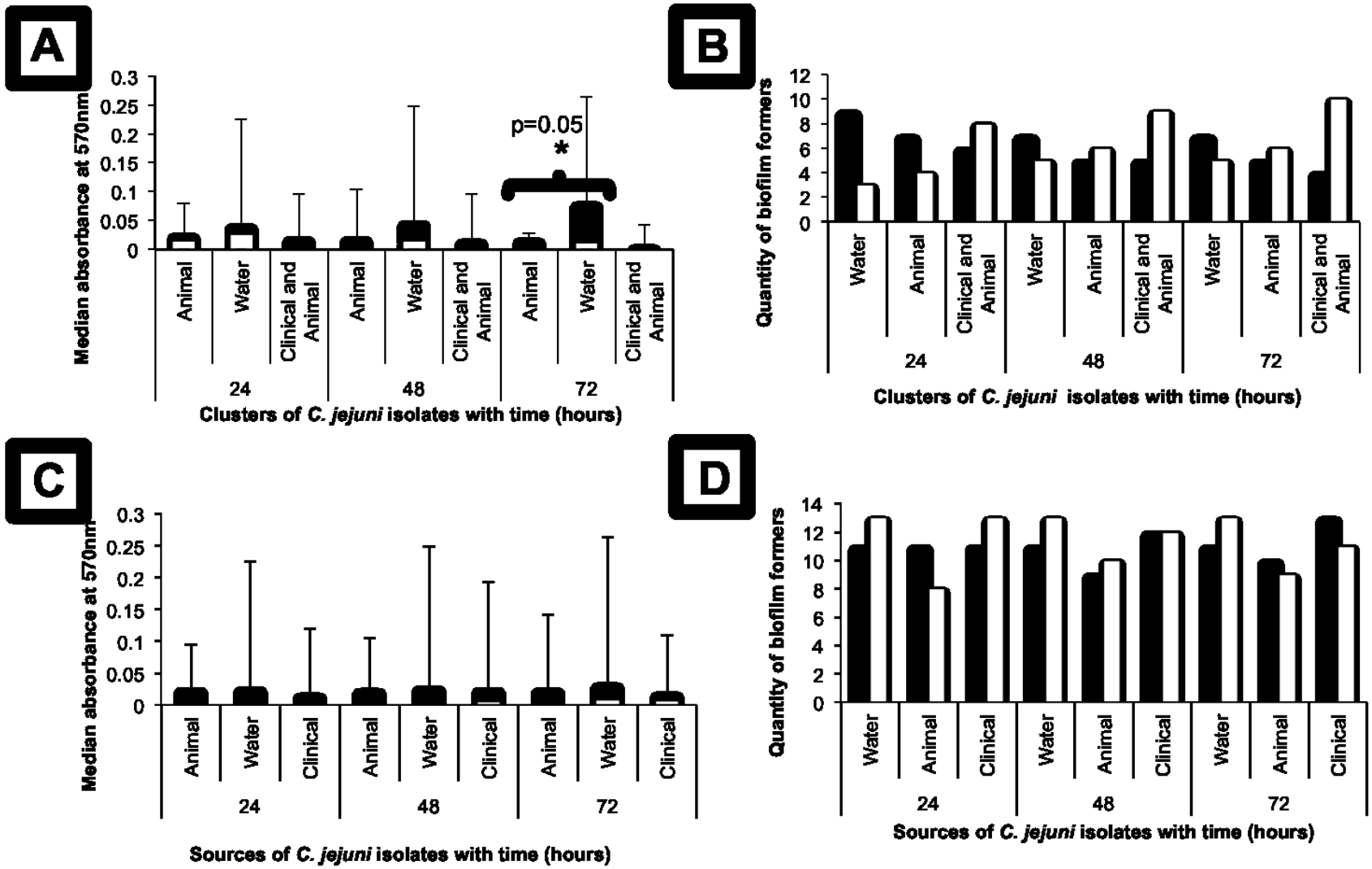
3.2.2.3 Biofilm formation of *Campylobacter* strains

Biofilms offer protection from stresses including antibiotics, sanitizers, heat stress, desiccation and immune responses, thus promoting survival of the bacteria in different environments (56, 71, 149). To compare *Campylobacter* strains from varied sources for their ability to produce biofilms, the biofilm-forming potential of isolates from clinical, animal and environmental reservoirs was assessed (Figure 6).

A total of 67 strains from varied sources were analyzed for their ability to form biofilms. Median absorbance measurements at the three time points were determined for strains sorted by cluster (Figure 6A) and by source (Figure 6C). Statistically significant differences between isolates separated in cluster categories (Figure 6A) were determined at the three time points. After 24 h, water clusters had the highest median biofilm formation, but the differences were not statistically significant when compared to clusters of clinical and animal and mostly animal isolates. There were no significant differences observed after 48 h, but strains in the water clusters had a high distribution and range of data. Biofilm formation at 72 h was significantly higher for water strains compared to clusters of mostly animal strains (Mann-Whitney test; $p=0.05$).

When sorted by source (Figure 6C), strains from water, animal and clinical sources had similar biofilm formation at 24, 48 and 72 h time points (Mann-Whitney test; $p>0.05$). The median absorbance readings for most of the strains were zero, indicating no biofilm formation. For every time point, the highest absorbance readings were for strains from water sources as demonstrated by the errors bars indicating the range of data.

Figure 6. Biofilm formation of *C. jejuni* isolates from clinical, animal and water sources. Biofilm formation of 67 strains from varied sources was tested after 24, 48 and 72 h incubation. Bacterial cultures were stained with crystal violet dye and the median absorbance was calculated from 4 replicates on each plate. Each experiment was repeated three times for the three time points and the data was presented in box plots (Figure 6A and 6C). For each group of strains, the box, which captures the interquartile range is split by the median line and is flanked by vertical lines representing the interval between the lowest (bottom) and the highest (top) values. Strains separated in clusters of animal (N=11), water (N=12) as well as clinical and animal (N=15) isolates, were statistically analyzed and box plots were created (Figure 6A). Water clusters had significantly higher biofilm formation than animal clusters after 72 h of incubation ($p = 0.05$). For strains compared by clinical (N=24), animal (N=24) and water (N=19) sources (Figure 6C), similar biofilm formation was observed after 24, 48 and 72 h. The number of biofilm-formers (Black bars) and non-biofilm-formers (White bars) varied among clusters (Figure 6B) and sources (Figure 6D), but no significant differences were observed.

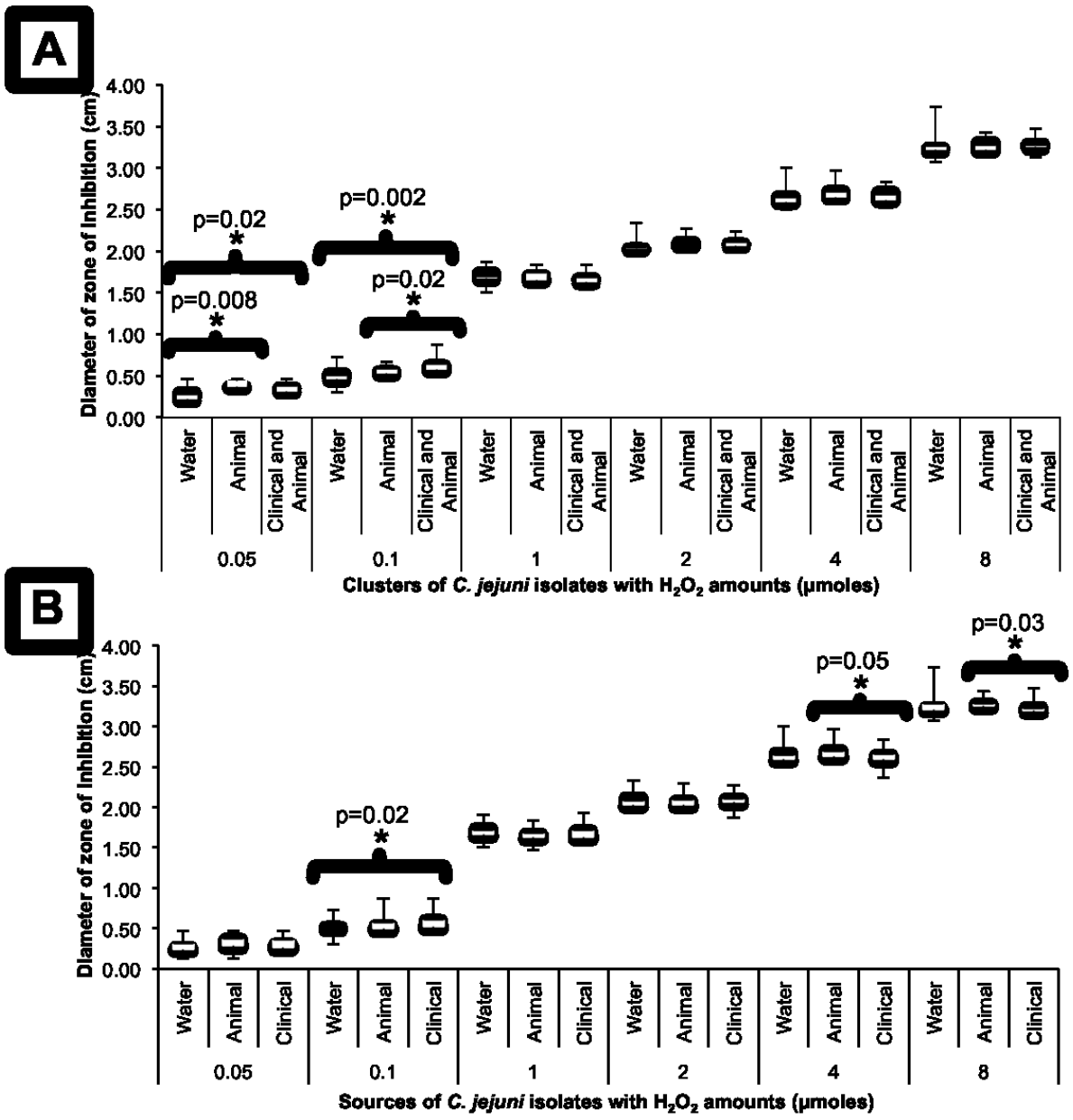

A
Median absorbance at 570nm
0.3
0.25
0.2
0.15
0.1
0.05
0
p=0.05
*
Animal
Water
Clinical and Animal
24
48
72
Clusters of C. jejuni isolates with time (hours)
B
Quantity of biofilm formers
12
10
8
6
4
2
0
Water
Animal
Clinical and Animal
24
48
72
Clusters of C. jejuni isolates with time (hours)
C
Median absorbance at 570nm
0.3
0.25
0.2
0.15
0.1
0.05
0
Animal
Water
Clinical
24
48
72
Sources of C. jejuni isolates with time (hours)
D
Quantity of biofilm formers
14
12
10
8
6
4
2
0
Water
Animal
Clinical
24
48
72
Sources of C. jejuni isolates with time (hours)

To identify the number of biofilm-forming strains (Figure 6B and 6D), median absorbance measurements of strains were compared with the median values of the non-biofilm-forming control (MH broth). Strains were classified as being biofilm formers if the median absorbance for the strains was significantly higher than the median value for the control as determined with the Student T-test. Quantity of *C. jejuni* biofilm-formers, after 24, 48 and 72 h incubation, differed between clusters (Figure 6B). The elevated numbers of strains that were not biofilm formers (Figure 6B) explained the near zero box plot distributions (Figure 6A). No significant differences observed when comparing quantities of biofilm formers and non-biofilm formers between clusters (Figure 6B) and sources (Figure 6D) at each time point (Chi Square test; $p>0.05$). Water clusters had the highest quantity of biofilm-formers at 24, 48 and 72 h, while clusters of clinical and animal had the most non-biofilm-formers at the three time points.

3.2.2.4 Hydrogen peroxide sensitivity of *Campylobacter* strains

Campylobacter spp. encounter hydrogen peroxide in environmental waters (98) and in the gut when it is released by phagocytic cells and bacteria present in the intestine (143). *Campylobacter* spp. in chicken may encounter hydrogen peroxide released by the chick immune system or other bacteria. To assess the sensitivity of strains from clinical, animal and water sources to hydrogen peroxide, assays were conducted for 67 *Campylobacter* strains (Figure 7). Sensitivity to increasing concentrations of hydrogen peroxide (0.05, 0.10, 1, 2, 4 and 8 µmoles) was determined for strains sorted by cluster (Figure 7A) and source (Figure 7B).

Figure 7. Sensitivity of clinical, animal and water *C. jejuni* isolates to hydrogen peroxide. Sixty-seven isolates, separated either into clusters of water (N=12), animal (N=11) as well as clinical and animal (N=15) strains (Figure 7A) or clinical (N=24), animal (N=24) and water (N=19) sources (Figure 7B), were tested for their sensitivity to 0.05, 0.1, 1, 2, 4 and 8 µmoles of hydrogen peroxide using a disc inhibition assay. A smaller zone of inhibition represented a stronger resistance to hydrogen peroxide. The mean diameter of zone of inhibition for each strain was calculated from three independent experiments and results were represented in box plots. For each group of strains, the box, which captures the interquartile range is split by the median line and is flanked by vertical lines representing the interval between the lowest (bottom) and the highest (top) values. *C. jejuni* isolates present in animal and clinical clusters were more sensitive to lower levels of hydrogen peroxide (Figure 7A). Sensitivity to higher amounts of hydrogen peroxide was greater for *C. jejuni* strains from animal sources when compared to clinical isolates (Figure 7B).


A
Diameter of zone of Inhibition (cm)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
p=0.02
p=0.002
p=0.02
p=0.008
Water
Animal
Clinical and Animal
0.05
0.1
1
2
4
8
Clusters of C. jejuni isolates with H2O2 amounts (µmoles)
B
Diameter of zone of Inhibition (cm)
p=0.03
p=0.05
p=0.02
Water
Animal
Clinical
0.05
0.1
1
2
4
8
Sources of C. jejuni isolates with H2O2 amounts (µmoles)

Significant variability in hydrogen peroxide resistance was observed among strains from different sources when analysed by clusters (Figure 7A). In the presence of 0.05 µmoles hydrogen peroxide, water strains were significantly less sensitive than animal strains (Mann-Whitney test; p=0.008) and isolates in clusters of animal and clinical strains (p=0.02). Isolates in clinical and animal clusters were significantly more sensitive to 0.10 µmoles of hydrogen peroxide than animal strains (p=0.02) and water strains, (p=0.002) while water isolates were less sensitive than animal strains (p=0.06). When exposed to 2 µmoles of hydrogen peroxide, water strains were less sensitive than isolates in animal clusters (p=0.06) and clusters of animal and clinical strains (p=0.06). Strains in water clusters had the highest resistance to low levels of hydrogen peroxide.

Clinical (n=24), water (n=19) and animal (n=24) were compared by sources for sensitivity to hydrogen peroxide (Figure 7B). Water strains were significantly less sensitive than clinical strains (Mann-Whitney test; p=0.02) when using 0.1 µmoles of hydrogen peroxide. When exposed to 4 or 8 µmoles of hydrogen peroxide, animal strains were significantly more sensitive than clinical strains with p values of 0.05 and 0.03, respectively. Water strains were more sensitive than clinical strains but the differences was not significant in the presence of 4 (p=0.4) or 8 µmoles (p=0.2) of hydrogen peroxide.

3.2.2.5 Adhesion and invasion of *C. jejuni* isolates

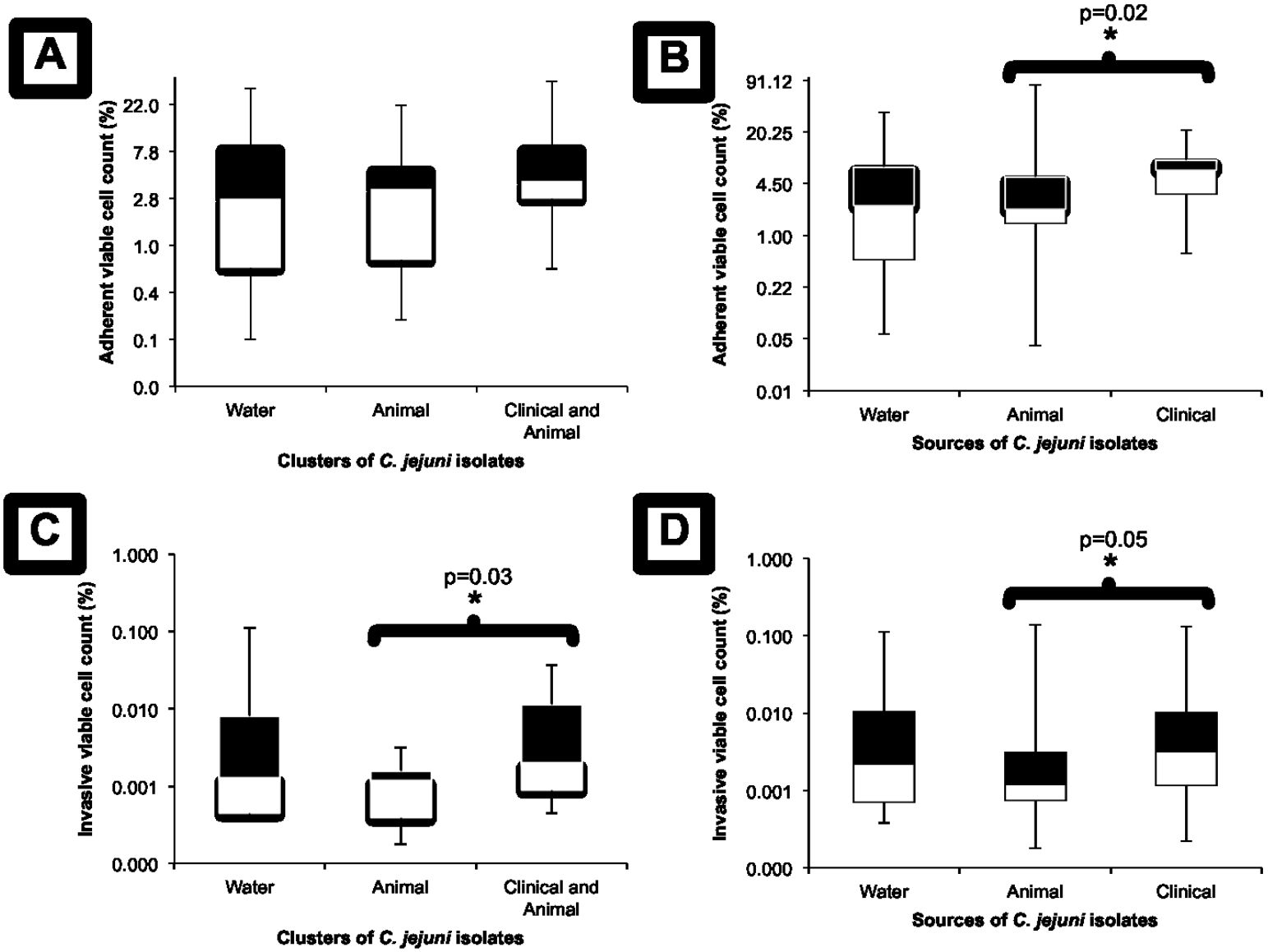
In clinical cases of campylobacteriosis, the bacteria colonize human intestinal epithelial cells and cause infection (67). Adhesion and invasion assays with the

human Caco-2 cell line are commonly performed to assess the virulence potential of *Campylobacter* strains.

The adherence and invasiveness of *Campylobacter* isolates from clinical, animal and water sources (Figure 8) to undifferentiated Caco-2 epithelial cells was compared by assessing the mean number of adherent (Figures 8A and 8B) and invasive bacterial cells (Figures 8C and 8D) for each strain. Significant differences were determined for strains grouped by source (animal, water, clinical) or cluster (animal, water, animal/clinical).

Adherence and invasion was significantly higher in clinical strains, compared to animal strains when strains were sorted by clusters or sources. Animal isolates were significantly less adherent than clinical strains (Mann-Whitney test; $p=0.02$). Invasion was significantly higher for strains in clusters containing clinical and animal isolates than strains in clusters of mostly animal isolates ($p=0.03$). When strains were compared by source, *C. jejuni* animal isolates were significantly less invasive than clinical strains ($p=0.05$), while isolates from water sources appeared to be more invasive than animal strains but the difference was not significant ($p=0.09$). Water and animal strains showed a high level of variability in both adherence and invasion, and the clinical strains had the smallest distribution. Clinical and water strains did not differ in adherence and invasion of Caco-2 cells. *Campylobacter* spp. adherence to Caco-2 human cells was not significantly different for strains sorted by clusters.

Figure 8. Adherence and invasion of *Campylobacter* isolates from clinical, animal and water sources. Sixty-seven isolates from clinical, animal and water sources were tested for their adherence and invasiveness to Caco-2 cells. *Campylobacter* strains were grown microaerobically and placed on a monolayer of undifferentiated Caco-2 cells. After 2 h incubation, cells were washed and total number of bacterial cells was determined by plating on CCDA plates. Following gentamicin treatment, the quantity of invasive bacteria was obtained. Subtracting the total amount of bacterial cells by the number of invasive bacteria yielded the quantity of adherent bacteria for each strain. Mean adherent viable cell counts of three independent experiments were plotted for strains sorted by clusters of clinical and animal (N=15), water (N=12) and animal (N=11) isolates (Figure 8A) and clinical (N=24), animal (N=24) and water (N=19) sources (Figure 8B). Viable cell counts are represented in percentage values using box plots. For each group of strains, the box, which captures the interquartile range is split by the median line and is flanked by vertical lines representing the interval between the lowest (bottom) and the highest (top) values. Strains from clinical sources were significantly more adherent than isolates from animal sources (p=0.02). Box plots of mean invasive viable cell count from three independent experiments were created for isolates separated by clusters (Figure 8C) and by sources (Figure 8D). Strains in animal clusters were less invasive than isolates in clinical and animal clusters (p=0.03). Clinical isolates were significantly more invasive than strains from animal sources (p=0.05).


A
Adherent viable cell count (%)
22.0
7.8
2.8
1.0
0.4
0.1
0.0
Water
Animal
Clinical and Animal
Clusters of C. jejuni isolates
B
p=0.02
*
Adherent viable cell count (%)
91.12
20.25
4.50
1.00
0.22
0.05
0.01
Water
Animal
Clinical
Sources of C. jejuni isolates
C
Invasive viable cell count (%)
1.000
0.100
0.010
0.001
0.000
p=0.03
*
Water
Animal
Clinical and Animal
Clusters of C. jejuni isolates
D
p=0.05
*
Invasive viable cell count (%)
1.000
0.100
0.010
0.001
0.000
Water
Animal
Clinical
Sources of C. jejuni isolates

3.2.2.6 Combining results for phenotypic assays

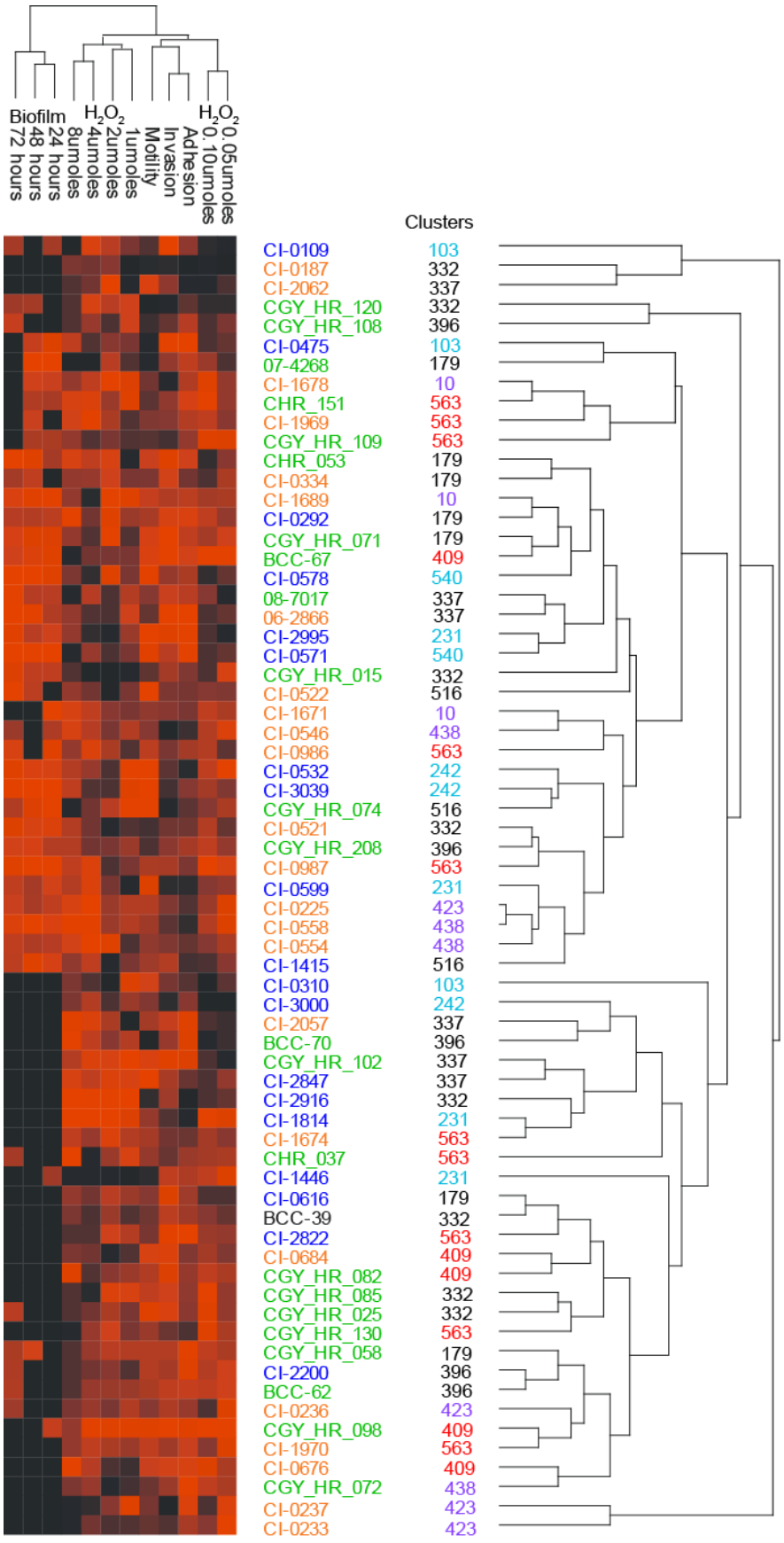
To assess phenotypic similarities between strains from varied sources and clusters, results were compared for motility, biofilm formation, adhesion, invasion and sensitivity to hydrogen peroxide. A hierarchical clustering map was created with the Cluster 3.0 program (Human Genome Center, University of Tokyo) using the Pearson's correlation (uncentered) similarity metric (Figure 9). The data from all the assays was combined to depict similarities between strains from varied sources and clusters. Experimental data were ranked from highest to lowest for each phenotypic experiment that was done.

While clinical, animal and water strains had high and low values for the phenotypic assays, isolates from clinical and animal sources were closer to each other, indicating greater similarities between them. A portion of isolates clustered based on CGF results and phenotypic data indicating that genetic relatedness could predict phenotypic similarities.

When comparing phenotypic assays, results for biofilm formation at 24, 48 and 72 h clustered together indicating similar differences between strains from varied sources. Also, there was separation of strains as those that did not form biofilms clustered together. This separation did not appear to be based on strain origin or genetic relatedness as the two groups of biofilm and non-biofilm formers contained isolates from varied sources and clusters.

Furthermore, the data obtained from the hydrogen peroxide sensitivity assays separated into two clusters when analyzed with the Cluster program. Clusters consisted of either the high or the low amounts of hydrogen peroxide. This was

Figure 9. Phenotypic analysis of *Campylobacter* strains from clinical, animal and water sources. Genetic relatedness of 67 strains was determined by Comparative Genomic Fingerprinting analysis by organizing them into clusters (10 103, 179, 242, 231, 332, 337, 396, 409, 423, 438, 516, 540 or 563). In this study, Isolates from clinical (Green), animal (Orange) and water (Blue) sources were compared according to source and clusters of mostly animal strains (10, 423, 438; Purple), water isolates (103, 231, 242; Light Blue) as well as clinical and animal isolates (409, 563; Red). Motility, biofilm formation (24, 48, 72 h), sensitivity to hydrogen peroxide (0.05, 0.10, 1, 2, 4, 8 µmoles), adhesion and invasion were assessed for all strains. Values were ranked from highest to lowest for all phenotypic assays. Using the Cluster 3.0 program (Human Genome Center, University of Tokyo), strains and phenotypic experiments data were clustered according to the Pearson's correlation (uncentered) similarity metric.


Biofilm 72 hours
Biofilm 48 hours
Biofilm 24 hours
H2O2 8umoles
H2O2 4umoles
H2O2 2umoles
H2O2 1umoles
Motility
Invasion
Adhesion
H2O2 0.10umoles
H2O2 0.05umoles
Clusters
CI-0109 103
CI-0187 332
CI-2062 337
CGY_HR_120 332
CGY_HR_108 396
CI-0475 103
07-4268 179
CI-1678 10
CHR_151 563
CI-1969 563
CGY_HR_109 563
CHR_053 179
CI-0334 179
CI-1689 10
CI-0292 179
CGY_HR_071 179
BCC-67 409
CI-0578 540
08-7017 337
06-2866 337
CI-2995 231
CI-0571 540
CGY_HR_015 332
CI-0522 516
CI-1671 10
CI-0546 438
CI-0986 563
CI-0532 242
CI-3039 242
CGY_HR_074 516
CI-0521 332
CGY_HR_208 396
CI-0987 563
CI-0599 231
CI-0225 423
CI-0558 438
CI-0554 438
CI-1415 516
CI-0310 103
CI-3000 242
CI-2057 337
BCC-70 396
CGY_HR_102 337
CI-2847 337
CI-2916 332
CI-1814 231
CI-1674 563
CHR_037 563
CI-1446 231
CI-0616 179
BCC-39 332
CI-2822 563
CI-0684 409
CGY_HR_082 409
CGY_HR_085 332
CGY_HR_025 332
CGY_HR_130 563
CGY_HR_058 179
CI-2200 396
BCC-62 396
CI-0236 423
CGY_HR_098 409
CI-1970 563
CI-0676 409
CGY_HR_072 438
CI-0237 423
CI-0233 423

expected as phenotype results demonstrate differences between source and cluster groups with regards to varying amounts of hydrogen peroxide.

In addition, results for motility, adhesion and invasion assays clustered together indicating that these phenotypes were related for most of the *Campylobacter* isolates from clinical, animal and water sources.

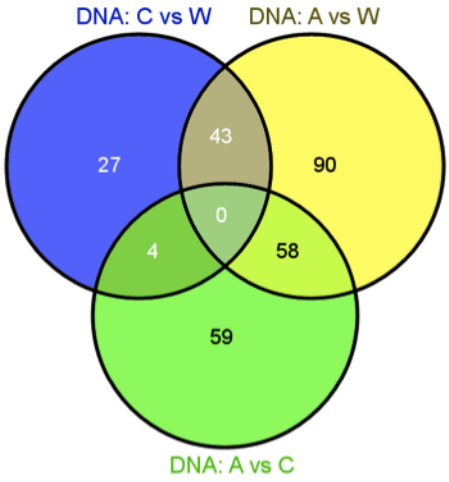
3.2.3 Microarray analysis

A whole genome *C. jejuni* NCTC 11168 DNA microarray was used to determine differences in genes expressed (RNA) or present (DNA) between clinical, animal and water sources. Two channel microarrays were performed for each strain with labelled complementary DNA and genomic DNA. The intensity values of replicate gene spots on arrays were mean-normalized. A ratio of raw intensity over background above 2 was considered expressed (RNA channel) or present (DNA channel). Strains from each source (clinical, animal and water) were grouped together to determine the number of strains in each source that expressed or contained each gene. Statistically significant differences for the presence (or expression) of a gene between “Animal vs Clinical”, “Animal vs Water” or “Clinical vs Water” sources was determined using the Fisher exact test. From each comparison, a list of genes was obtained (Tables C1 to C3 and C5 to C8). The number of genes present (DNA) and expressed (RNA) for clinical, animal and water sources was illustrated in Venn diagrams to identify similarities (Figure 10).

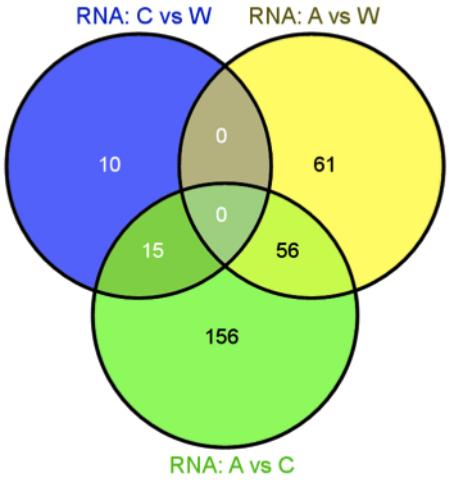
Fourteen animal, 12 water and 13 clinical strains were analysed by microarray. Isolates were selected from all clusters to observe genes expressed and present in strains from varied sources, regardless of their genetic relatedness.

Figure 10. Similarities in genes present or expressed between "Animal vs Clinical", "Animal vs Water" and "Clinical vs Water" source groups. There were no genes commonly present (DNA) in "DNA: C vs W" (N=74), "DNA: A vs W" (N=191) and "DNA: A vs C" (N=121) (Figure 10A). Fifty-eight genes were present in both "DNA: A vs W" and "DNA: A vs C", 43 were in both "DNA: C vs W" and "DNA: A vs W", and 4 were in both "DNA: C vs W" and "DNA: A vs C". The numbers of genes expressed (RNA) in the three sources groups were compared and there were no genes present in all three groups (Figure 10B). Fixty-six genes were commonly found in "RNA: A vs W" (N=117) and "RNA: A vs C" (N=227) groups while 15 were in both "RNA: C vs W" (N=25) and "RNA: A vs C".

A


DNA: C vs W
DNA: A vs W
27
43
90
0
4
58
59
DNA: A vs C

B


RNA: C vs W
RNA: A vs W
10
0
61
0
15
56
156
RNA: A vs C

3.2.3.1 Gene content

The presence of genes in strains from varied sources was also compared in a Venn diagram (Figure 10A). There were 74 genes (Table C1) that were significantly different among the clinical and water isolates (Figure 10A, Blue circle), with 6 genes (CJE0273, CJE1550, CJE1732, *Cj0011c*, *Cj0033*, *Cj1248*, *Cj1642*) significantly associated with water strains and 68 genes, which were more prevalent in clinical strains. All of the 191 genes present that differed significantly in their presence among among animal and water isolates (Figure 10A, Yellow circle) had a higher prevalence in animal strains as compared to water isolates (Table C2). The 121 genes differing significantly among animal and clinical strains (Figure 10A, Green circle) were all more commonly found in animal strains as compared to clinical strains (Table C3). There were 58 genes present in both the “DNA: A vs W” and “DNA: A vs C” groups, 43 genes between the “DNA: C vs W” and “DNA: A vs W” groups and 4 genes between the “DNA: C vs W” and “DNA: A vs C” groups (Figure 10A).

The presence of phage genes for strains from clinical, animal and water sources was compared for *Campylobacter jejuni* integrated elements (CJIE). Strain RM1221 is similar to strain NCTC11168 with the exception of 4 insertions. These include CJIE1 (*CJE0213-CJE0273*), CJIE2 (*CJE0544-CJE0601*), CJIE3 (*CJE1092-CJE1155*) and CJIE4 (*CJE1418-CJE1474*) (38). CJIE1, also called *Campylobacter* Mu-Like Prophage 1 (CMLP1), encodes a prophage while CJIE3 is an integrated plasmid. CJIE2 and CJIE4 contain genes expressing phage-related endonucleases, methylases or repressors (38, 115). To identify clinical, animal and water strains

containing phage, microarray results were used to determine presence of genes belonging to CJIE1, CJIE2, CJIE3 and CJIE4 insertions (Table C4). CJIE1 Mu-like prophage genes were present in 79% of animal, 62% of clinical, and 42% of water isolates A hierarchal clustering map of genes present in the *C. jejuni* integrated elements was created to demonstrate the absence of genes (Black), as well as higher (Green) or lower (Red) gene expression levels relative to gene content (Figure C1).

3.2.3.2 Transcript profiles

Data were also analysed by considering if genes were both present and expressed (DNA value was greater than 2x background and RNA value was greater than two 2x background) among different sources (Figure 10B). In the “RNA: C vs W” group (Figure 10B, Blue circle), 25 expressed genes were significantly associated with either the clinical or water strains (Table C5). Four of these genes were more prevalent in water strains (*Cj0196c*, *Cj0203*, *Cj0264c* and *Cj0436*), while 21 were more often found in clinical isolates. In the “RNA: A vs W” group (Figure 10B, Yellow circle), all of the 117 genes were in a higher number of animal strains as compared to isolates from water sources (Table C6). The “RNA: A vs C” group (Figure 10B, Green circle) consists of 227 genes with expression being greater in the animal isolates as compared to the clinical strains (Table C7). Among the genes identified, 56 were common in the “RNA: A vs W” and “RNA: A vs C” groups. When comparing the lists in “RNA: C vs W” and “RNA: A vs C”, 15 genes were similar (Figure 10B).

A Student's T-test was done to identify genes with significantly different abundance levels of transcripts among source groups (Tables C8 and C9). The relative abundance of transcripts was determined for every gene by dividing the normalized value of expression (RNA) by the value of presence (DNA). The median of relative abudnace of every gene in a source was calculated from data of strains in each source group (Clinical, animal, water). The data was sorted to show only genes that were significantly different using the T-test (p-value <0.01) and that had at least two fold differences between the source groups. Two genes were up-regulated in clinical strains when compared to water isolates, while isolates in the animal source group had 18 genes with higher abundance in transcripts relative to the clinical source group and 58 genes when compared to the water source group (Table C8). Strains in clinical source groups had two genes down-regulated relative to water isolates while the animal source group had 1 genes with significantly lower abundance in transcripts when compared to the clinical source group and 5 genes relative to the water source group (Table C9).

To identify similarities in gene expression between strains, clustering of relative expression of genes (RNA/DNA) was performed using the Cluster 3.0 program (25). Genes and microarrays were normalized and hierarchical clustering was done using the Pearson's correlation (uncentered) similarity metric. A phylogenetic tree indicated the relationship of strains determined by microarray analysis (Figure C2). Results from duplicate arrays clustered together indicating reproducibility of methods.

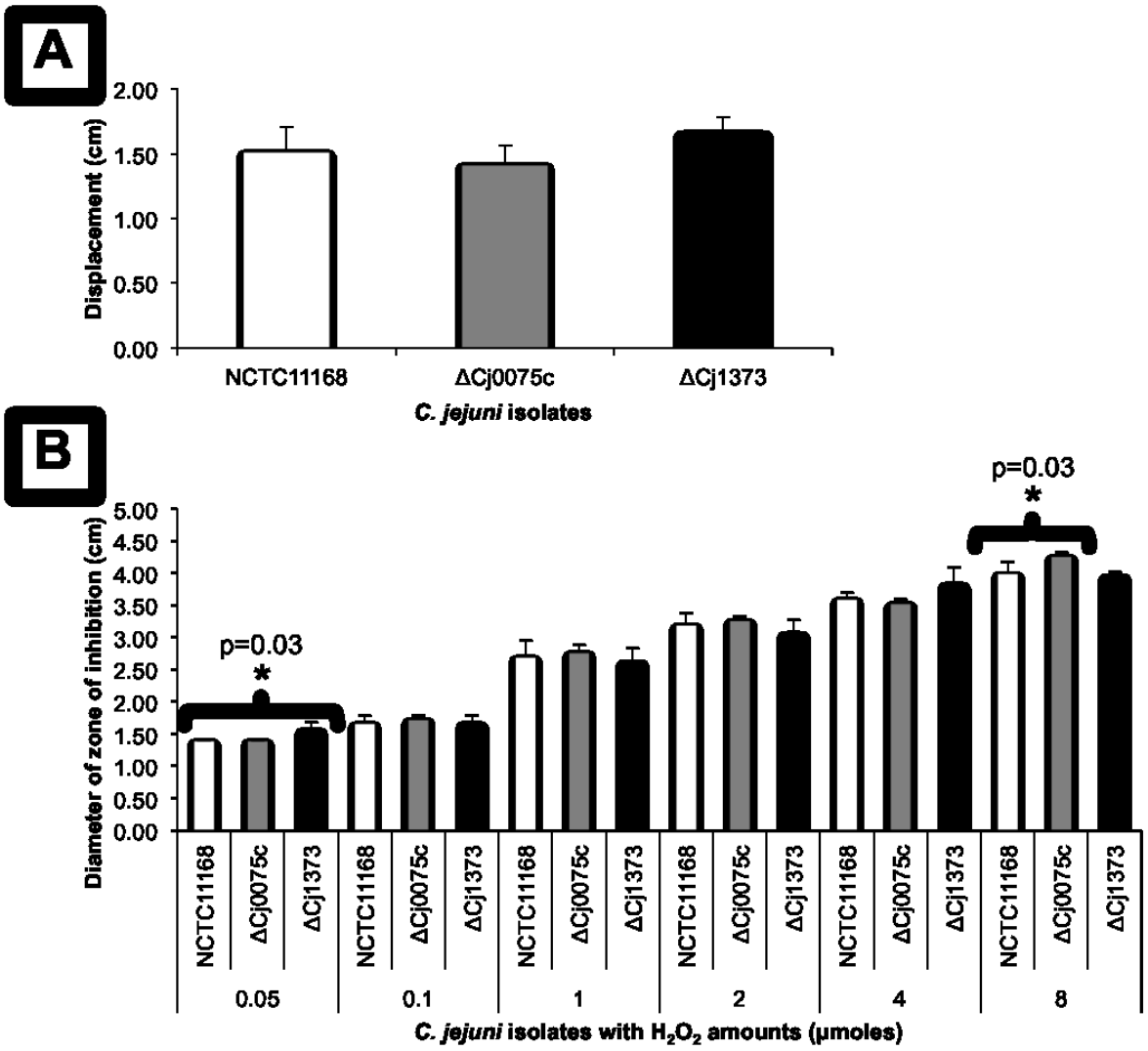
3.3 *C. jejuni* mutant strain analysis

Based on results from previous comparative genomic studies completed in the *Campylobacter* lab at the Bureau of Microbial Hazards, 5 genes including *Cj0075c*, *Cj0199c*, *Cj0305c*, *Cj0809c* and *Cj1373*, were more highly associated with clinical strains of *C. jejuni*. To assess the role of these genes in *C. jejuni*, the construction of mutants for these 5 genes was attempted. Two mutant strains ΔCj0075c and ΔCj1373 were successfully constructed by disrupting the respective genes with a chloramphenicol cassette in *C. jejuni* NCTC11168 strain. For genes *Cj0199c*, *Cj0305c* and *Cj0809c*, plasmids containing the disrupted genes were constructed but attempts to naturally transform the plasmids into *C. jejuni* to produce the mutants were unsuccessful.

The *Cj1373* gene, 2472 bp in length, encodes an integral membrane protein. The *Cj0075c* gene, 741 bp in length, expresses one of the three subunits of a non-flavin iron-sulfur oxidoreductase (Cj0075c-73c). This latter is one of two enzymes, including a flavin and iron-sulfur oxidoreductase (Cj1585c), that are important for the growth of *C. jejuni* NCTC11168 on L-lactate (150). Only a double mutant of these two genes stops the growth of *C. jejuni* on L-lactate (150). To determine the importance of *Cj0075c* and *Cj1373* genes on *C. jejuni* virulence, motility and sensitivity to hydrogen peroxide were assessed for the wild-type strain NCTC11168 and the two mutants, ΔCj0075c and ΔCj1373 (Figure 11).

Mean displacements were plotted based on results from three independent experiments and error bars represented the standard deviation (Figure 11A). No statistically significant differences were observed for the mean displacement of the

Figure 11. The importance of *Cj0075c* and *Cj1373* genes for *C. jejuni* motility and sensitivity to hydrogen peroxide. Motility assays were performed for the wild-type NCTC11168 (White Bars) and mutant strains, ΔCj0075c (Grey Bars) and ΔCj1373 (Black Bars), by stabbing 1 µL suspensions of overnight cultures into 0.4% MH agar plates. The radius of the circle created by bacterial movement was measured and mean displacements of three independent experiments were plotted with error bars indicating standard deviation (Figure 11A). Genes *Cj0075c* and *Cj1373* did not directly influence motility in *C. jejuni*. Sensitivity to hydrogen peroxide was also tested for the three strains by diluting overnight culture and plating on new MHA plates. Discs containing 0.05, 0.1, 1, 2, 4 and 8 µmoles of hydrogen peroxide were placed on the lawns of bacteria. Mean diameters of zone of inhibition were calculated from three independent experiments and results were plotted with errors bars representing standard deviation (Figure 11B).


A
Displacement (cm)
2.00
1.50
1.00
0.50
0.00
NCTC11168
ΔCj0075c
ΔCj1373
C. jejuni isolates
B
Diameter of zone of inhibition (cm)
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
p=0.03
*
p=0.03
*
NCTC11168
ΔCj0075c
ΔCj1373
0.05
0.1
1
2
4
8
C. jejuni isolates with H2O2 amounts (µmoles)

wild-strain NCTC11168 with that of ΔCj0075c (Student T-test, p=0.2) and ΔCj1373 (p=0.09) mutants. The ΔCj0075c mutant had the lowest motility while ΔCj1373 had the highest motility among the three strains.

Mean diameters of zone of inhibition from three independent experiments of hydrogen peroxide sensitivity assays were plotted for each strain (Figure 11B). A larger diameter indicated a higher sensitivity to hydrogen peroxide. ΔCj1373 was significantly more sensitive to 0.05 μmoles hydrogen peroxide than the wild-type strain (Student T-test, p=0.03). With the addition of 8 μmoles hydrogen peroxide, the mutant strain ΔCj0075c was significantly less resistant than the wild-type strain (p=0.03). *Cj1373* played a role in resistance of *C. jejuni* at lower concentrations while *Cj0075c* did at higher amounts of hydrogen peroxide.

4: DISCUSSION

4.1 Impact of phage on virulence of *C. jejuni*

The phage-containing isolates (00-2425, 00-2538, 00-2544) differ from the control strain (00-2426) due the insertion of *Campylobacter* Mu-Like Prophage 1 (CMLP1) also called CJIE1. The three phage-containing isolates were previously found to be more adherent and invasive in INT-407 cells than the control strain (19). Increased motility that was observed (Figure 2A) could help to explain this difference, as more motile strains would have a greater ability to reach the human epithelial cell layer where they can adhere and invade to cause infection.

Little is known about the impact of prophage on *Campylobacter* motility, and conflicting results on this subject have been observed with bacteria from other genera. The deletion of the CP4-57 prophage in *E. coli* BW25113 strain led to an 8-fold increase in motility (153). In contrast, in *Pectobacterium atrosepticum*, responsible for bacterial soft rot of potatoes, the deletion of the prophages ECA41 or ECA29 created a significantly less motile strain when compared to the wild-type (32). Motility in other genera varied with the prophage type and the host bacterium. We observed an increase in motility, but it is unclear whether this is specifically due to the presence of the prophage, or to unrelated variability in *Campylobacter* motility.

When comparing the four strains for sensitivity to hydrogen peroxide, variability was observed (Figure 2B), indicating that there are other factors influencing sensitivity to this oxidant. Phage insertions could be responsible for

inducing differential expression of host genes, thereby impacting phenotypic traits. Moreover, gene content and expression of the host bacteria may modulate the role that a phage could have on the host cell. The effects of oxidative stress may be more evident when comparing gene expression profiles of phage-containing isolates and the control strain in environments with and without hydrogen peroxide.

The impact of prophage on bacterial cells in the presence of hydrogen peroxide has been described for other genera. Hydrogen peroxide induces Shiga toxin-converting prophage in *E. coli* O157:H7, leading to the production and the secretion of Shiga toxins (82). The release of these toxins increases pathogenicity of the bacterial strain by causing apoptosis of the host cell (132). Similarly, hydrogen peroxide induces the prophage *Gifsy-2* in *Salmonella* Typhimurium (35). This prophage expresses the *sodC* gene, encoding for a superoxide dismutase that protects bacterial cells against macrophages and thereby increases virulence of the bacteria (35). In another *E. coli* study, a deletion in the prophage CPS-53 led to a decrease in hydrogen peroxide sensitivity (153).

The influence of prophages on biofilms has been studied in several genera of bacteria. In *E. coli* BW25113, which naturally contains 9 prophages, removal of all prophage resulted in the inability to form biofilms (153). This was thought to be due to effects of reduced cell aggregation in these *E. coli* mutants. In our study, the three phage-containing isolates were not significantly better biofilm formers when compared to the control strain (Figure 3). The presence of phage influenced biofilm formation differently for the three phage-containing isolates (00-2425, 00-2538, 00-2544). Further testing of aggregation in phage-containing *C. jejuni* isolates may

explain observed differences in biofilm formation between strains in this study. Also, due to the variability in results, the crystal violet biofilm protocol will require further optimization to address replicate variability. In the future, the presence of biofilms produced by each strain could be further confirmed using an electron microscope.

To determine the impact of *Campylobacter* phage insertions on motility, sensitivity to hydrogen peroxide and biofilm formation, a mutation or deletion of the prophage from the three phage-containing isolates could be done or a greater number of strains containing prophage can be phenotypically analysed using more control isolates.

Gene expression profiles of phage-containing isolates and the control strain identified several genes that were found up-regulated in the presence of phage (Table 1). Genes *ilvD* (Cj0013), *trpE* (Cj0345) and *metC'* (Cj1392) play functional roles in amino acid biosynthesis, while *Cj1099* is important for degradation of macromolecules. A number of genes, including *rpmI* (Cj0244), *rplT* (Cj0245), Cj0311, *rpmB* (Cj0450c), *rplA* (Cj0475), *rpsG* (Cj0492), *rpsR* (Cj1072), *rpmJ* (Cj1591), *rplQ* (Cj1596), *rplR* (Cj1691c), *rplN* (Cj1697c), *rpmC* (Cj1699c) and *rplP* (Cj1700c), take part in ribosomal protein synthesis and modification. *Cj0035c* and *Cj0311* genes are important for drug sensitivity as well as antibiotic resistance. Gene *Cj0311* encodes the ribosomal protein *Ctc*, a general stress protein in *Bacillus subtilis* possibly required for effective translation under stressful conditions (118).

DNA replication, restriction, modification, recombination and repair genes such as *uvrA* (Cj0342c), *ssb* (Cj1071), *dnaG* (Cj1638) were up-regulated in phage-containing isolates. UvrA, part of the UvrABC DNA repair system, serves as a DNA-

binding protein as well as an ATPase and is important for the production of energy (88). Other genes involved in energy metabolism include *Cj1204*, encoding a proton gradient dependent ATPase, and *Cj0333c*, expressing a ferredoxin (43). Genes involved in protein translation, modification and secretion with increased expression in phage-containing isolates are *slyD* (Cj0115), *Cj0606* and *tsf*.

Higher transcript levels were observed for genes *Cj0177*, *Cj0178*, *pstS* (Cj0613), *Cj0919c*, *Cj1663* that encode transport and binding proteins. These genes have recently been shown to be important for colonization and pathogenicity of *C. jejuni*. The *phosR/S* phosphate uptake regulon is responsible for transcription regulation of the *pst* operon that includes *pstS*, a phosphate binding protein (29). The two component system *phosR/S* plays a role in the *Campylobacter* phosphate response (29). This response is important for flagella growth, quorum sensing and the expression of virulence associated genes (29). *Cj0919c* encodes an ABC-type amino-acid transporter permease that is part of the operon containing the *peb1A*, a gene that encodes a putative adhesion (36). The uptake of iron is required for *C. jejuni* to colonize and cause infection in the host (94, 114). Among the systems that have been studied in *C. jejuni*, the Cj0173c-Cj0178 system uptakes iron from ferri-transferrins (94). The *Cj0177* and *Cj0178* genes encode an iron transport protein and a TonB-dependent outer membrane receptor, respectively. *Cj1663* encodes an ATP-binding protein that is a component of the Cj1658-Cj1663 system responsible for iron uptake from ferri-rhodotorulic acid (94).

Cj0951c, encoding the methyl-accepting chemotaxis protein (MCP)-domain of the signal transduction protein Tlp7, is a gene shown to be important for invasion of

Caco-2 cells (148). This gene was highly expressed in phage-containing isolates when compared to the control strain. This may explain the increased adherence and invasion of the phage-containing strains, 00-2425, 00-2538 and 00-2544, relative to the control 00-2426 isolate (19, 20).

Microarray results for the phage-containing isolates and the control strain were not confirmed by real-time PCR experiments. For the analysis of microarray data, spot saturation was corrected using a high and low PMT scan of slides, which in turn increases the dynamic range of expression levels on a linear scale (30, 163). The high PMT scan would eliminate spots with intensity values below the appropriate range while saturating high intensity spots (83). This method allows the detection of smaller intensity spots that would be harder to correctly identify without increasing the PMT voltage (83). The low PMT scan would not include saturated spots. The intensity value of a spot can vary depending on the size and the shape of the spot and the distribution of hybridized fluorophores during scanning (30, 83). All of these properties can influence microarray data analysis and results for differentially expressed genes.

In the real-time PCR experiment, a high variability in Ct values obtained for biological replicates may have impacted determination of transcript levels. This may have been caused by RNA degradation or variability due to the passage of the strain as RNA isolation for the real-time PCR experiment was done after the microarray analysis was completed.

Issues surrounding possible problems with hybridization kinetics with unrelated probes could be resolved with more accurate full genome expression

analysis such as RNA sequencing. This latter method could determine with higher accuracy the expression of genes in the four isolates and could provide more specific information about the genome of each strain (104).

4.2 Clinical, animal and environmental *Campylobacter* isolates

Strains isolates from clinical (N=24), animal (N=24) and water (N=19) sources were compared using phenotypic and genetic experiments.

To determine doubling times for a select number of strains from each source group, growth curve experiments were conducted. Growth rate results (Figure 5A) for *Campylobacter* isolates from clinical, animal and water sources were consistent with published studies showing doubling times for *C. jejuni* strains ranging between 1.2 and 1.8 h (23, 75, 131). These strains were grown at 37°C to compare growth rates for strains under conditions similar to those inside the human host. The optimal growth temperature for *C. jejuni* is between 37 and 42°C (65, 75, 142). *C. jejuni* cells growing at 37°C had higher chemotactic responses than those at 42°C and there were differences in gene and protein expression between cells growing at these two temperatures (22, 65, 142). It was important to determine the growth rates of strains from clinical, animal and water sources to ensure phenotypic and genetic experiments were completed in the late logarithmic phase. Expression of *Campylobacter* virulence genes is strain dependent and varies with the growth phase (44). During the late logarithmic phase up to the early stationary phase, expression of virulence genes is observed when compared to the stationary phase, where some strains become more sensitive to stress (44).

A number of factors are known to influence the growth rates of *Campylobacter* strains. The genome of *C. jejuni* NCTC11168 encodes oxidases and reductases, proteins important for aerobic and anaerobic respiration, respectively (136). When limiting oxygen in microaerophilic conditions, the growth rates of *C. jejuni* decrease due to the inability of *C. jejuni* to synthesize DNA without oxygen (136). When adding fumarate, nitrate, TMAO or DMSO, which require very low levels of oxygen and reductases for respiration, growth rates increase (136). It would be interesting to compare growth rates of clinical, animal and water isolates in the presence or absence of oxygen or in nutrient-depleted environments. This might give insight into the conditions that promote survival of *Campylobacter* and its transmission between niches and hosts. For example, water strains of *Campylobacter* spp. are recovered from aerobic environments but to cause infection in humans, adaptation to microaerophilic environments is required. Identifying factors that alter or promote adaptation of strains from varied sources could be very important in determining the virulence potential of these strains.

4.2.1 Phenotypic analysis

Campylobacter isolates were compared by separating strains (Table B1) into clusters and sources to identify differences in phenotypic properties such as motility, biofilm formation, hydrogen peroxide sensitivity, adhesion and invasion.

For motility, strains in clusters (Figure 5B) of genetically related animal and clinical strains were more motile than isolates in the animal clusters alone. The three animal clusters were taken from larger pools of primarily animal strains with the exception of cluster 438 that also included a clinical strain. It would be of interest to

analyse a greater number of strains from the animal clusters to determine if they are significantly less motile than strains in animal and clinical clusters. This could indicate that animal strains that are more motile may be more likely to cause human infection.

Genetic fingerprints from CGF experiments were compared between the three cluster groups to identify differences in the presence or absence of hypervariable genes that may be linked to motility (Table B2). The majority of strains (9 of 15) in the clinical and animal clusters had genes present in the LOS locus, including *Cj1136*, *neuB1* (*Cj1141*) and *hldD* (*Cj1151c*), as well as *kpsS* (*Cj1431c*) and *glf* (*Cj1439c*) in the capsule locus. The *neuB1* (*Cj1141*) encodes a NANA (N-acetylneuraminic acid) synthetase important for LOS sialylation. Although mutation of *neuB1* was not found to directly affect motility of *C. jeuni*, sialylation of surface structures (LOS and flagella) is known to be important for virulence of the bacteria, especially for the development of Guillain-Barré Syndrome and Miller Fisher syndrome (81, 116). The *Cj1136*, *Cj1141*, *Cj1151c*, *Cj1431c* and *Cj1439c* genes were absent in all the strains present in animal clusters. LOS and CPS, important for virulence, vary in structure among *Campylobacter* isolates (69, 164). Perhaps the loss of LOS and capsule locus genes leads to less motile animal isolates. This is difficult to predict because in water clusters, most of the strains (10 of 12) lacked the LOS and capsule locus genes, but there was a lot of variation in the extent of motility in these strains.

Significant differences in motility were not observed for strains when compared by source (Figure 5C). These results were similar to a smaller scale study that was

done with 8 strains isolated from humans, broilers, milk, and bovine sources (49). These 8 strains, which were all identified as belonging to the MLST sequence type 21 (ST-21), had similar motility regardless of source (49). A comparative study with over 100 strains and an equal number of isolates from all sources and different sequence types, would be required to better understand the role that strain source plays on *C. jejuni* motility.

Using CGF results for strains organized by source (Table B3), the gene *htrB* (Cj1134), part of the LOS locus (*Cj1131c-Cj1152c*), was present in more animal strains (20 of 24) than clinical (14 of 24) or water (9 of 19) isolates. This gene was the only one among those linked to motility that varied among strains from different sources.

When comparing motility results from strains sorted by clusters (Figure 5B) and sources (Figure 5C), strains from animal sources and those in animal clusters displayed the smallest variability in motility. The distribution of data for strains from water sources (N=19) and those genetically related isolates grouped in water clusters (N=12) was similar. However, the median motility was lower for water strains analysed by source compared to clusters (Figure 5C). Isolates genetically clustered with animal and clinical strains might be less motile. To test this hypothesis, a larger number of strains would need to be compared for motility as 12 out of the 19 water isolates were in water only clusters and this was not an adequate number of strains to achieve sufficient statistical power. Strains from clinical sources (Figure 5C) had a wider dispersion of data when compared to isolates in clinical and animal clusters (Figure 5B).

Black et *al.* (11) illustrated the importance of bacterial motility for infection of human epithelial cells by showing that only motile *C. jejuni* variants were recovered in stool samples of human volunteers that had ingested motile and non-motile bacteria. In addition, a motile wild-type strain colonized the gastrointestinal tract of chicks at higher levels than a non-motile mutant strain. In our study, three clinical strains including BCC-70, 07-4268, CGY_HR_120 had very low motility with displacements below 0.85 cm but nevertheless they were found in stool samples. Motility, high or low, conferred the ability to invade host cells (50, 87). Whether these less motile strains caused less severe symptoms than more motile strains was not determined. For animal strains in our study, as most strains were collected from animal faeces, motility, high or low, contributed to intestinal colonization.

C. jejuni motility is increased in highly viscous environments, with speeds of up to 75 μm/second, promoting colonization of bacteria in the mucous lining of the human intestine (14, 50, 138). In the chicken model, chemotactic motility brings the bacterium to the gastrointestinal tract of the animal where amino acids are in abundance (116, 170). It would be of interest to test the motility of *C. jejuni* strains from animal, clinical and environmental sources when placing them in more viscous conditions and varying nutrient availability. This may explain differences in pathogenicity between strains from varied sources, as differing survival and virulence mechanism could be identified.

Biofilm formation for strains sorted by clusters (Figure 6A) and sources (Figure 6C) were similar in that isolates from water clusters and source groups had the highest variability in biofilm formation. The relative quantity of biofilm and non-

biofilm-formers between clusters (Figure 6B) and sources (Figure 6D) differed. For strains separated by source (Figure 6D), in each of the source groups, there was an equal number of biofilm-formers and non-biofilm formers. For strains organized in clusters (Figure 6B) the results varied, but water clusters tended to have more biofilm formers at every time point.

The biofilm formation protocol used in this study required optimizing, as well-to-well variations were observed in and between experiments. While the perfect biofilm formation experiment has yet to be identified, a number of *Campylobacter* biofilm methods have been reported. For example, Congo red dye can be used instead of crystal violet (129), and different materials for bacterial binding surfaces can be used such as glass or plastic (71). Furthermore, biofilm formation can vary with the type of culture broth (128), and the levels of environmental oxygen. Identifying a protocol yielding less variable data will enable more accurate quantification of biofilm formation in *Campylobacter*. It would also be beneficial to confirm biofilm growth using a scanning electron microscope.

Several bacterial species, including *Pseudomonas* spp., *Staphylococcus* spp., *E. coli*, *Bacillus* spp. and *Flavobacterium* spp., have the ability to form biofilms in the environment near animal facilities and *Campylobacter* can bind to them acting as a secondary colonizer (56, 149). *Campylobacter* biofilms in water promote transmission of the bacteria to animals and humans by protecting the bacteria and promoting their survival (71, 129). Formation of *C. jejuni* biofilms has not been observed *in vivo*, however experimental designs using static surfaces have shown it to be dependent on different growth conditions (50, 56). In a recent publication,

biofilm formation of *C. jejuni* NCTC11168 and its non-motile variant increased in aerobic conditions, when compared to microaerophilic conditions (129). For future experiments, biofilm formation of strains from clinical, animal and environmental sources could be compared under conditions with higher or lower oxygen, as well as with fluctuating temperatures. Changing oxygen levels could give insight into the mechanisms that *C. jejuni* uses to survive inside hosts and the environment. Examining biofilm formation at different temperatures could determine how quickly biofilms form under different growth conditions and how long they persist.

Increasing sensitivity with higher amounts of hydrogen peroxide was observed for strains separated by clusters (Figure 7A) and by sources (Figure 7B). Strains in water clusters had the largest variability in hydrogen peroxide sensitivity but when comparing water strains by sources, the range was only high relative to clinical and animal source groups, with 4 and 8 µmoles of hydrogen peroxide. No significant differences were observed with the addition of 1 and 2 µmoles of hydrogen peroxide. At concentrations below 1 µmoles, water strains of *Campylobacter* were the least sensitive but at concentrations above 2 µmoles, decreased sensitivity was observed among the clinical isolates. In theory, strains should display similar differences in sensitivity to hydrogen peroxide with the addition of all concentrations. As hydrogen peroxide diffuses through the bacterial lawn on the plate, the concentration will get lower as you move away from the disk that is placed at the centre of the plate. Therefore, when measuring sensitivity at all concentrations, it is expected to have the same differences. In our study, sensitivity does vary between strains with the addition of all concentrations however when

grouped by clusters and by source, sensitivity to hydrogen peroxide between groups does not fluctuate similarly. Although significant differences were observed at lower concentrations, results with higher concentrations of hydrogen peroxide are more reliable due to limitations in the ability to measure small differences with a ruler. Also, the range of measurements with addition of the 6 hydrogen peroxide concentrations is low indicating that strains in a group have similar resistance and that small differences between measurements would influence statistical analysis.

To identify differences in genetic fingerprints present in strains of water clusters that may have contributed to the decreased hydrogen peroxide sensitivity, CGF results were compared based on clusters (Table B2). The gene *Cj1585c*, encoding a FAD binding domain/putative oxidoreductase, was present in 2 (12 total) water strains, all of the strains in animal clusters (11 total), as well as the 15 isolates in the animal and clinical cluster (150). The *tonB1* (*Cj0181*) gene was found in 2 water isolates, 10 animal strains and all of the isolates in the animal and clinical cluster. The latter gene is a TonB transport protein that is part of the *Cj0179-Cj0181* complex (61). A common link was illustrated between flavine adenine dinucleotide (FAD), iron levels and hydrogen peroxide in *E. coli* (161). The reduced form of FAD, $FADH_2$, reacts with ferric iron to produce ferrous iron. In turn, the ferrous iron reacts with hydrogen peroxide to produce ferric iron, a hydroxyl anion and a hydroxyl radical. This hydroxyl radical oxidizes DNA and damages the host cell. In *Campylobacter*, ferrous iron activates Fur, which in turn blocks TonB expression. TonB would be activated to replenish the loss of iron (113). The expression of both *Cj0181* and *Cj1585c* may, consequently, promote the damage of the host cell in the

presence of hydrogen peroxide. These genes were absent in most strains from the water clusters (only 2 strains out of 12), possibly indicating their role in the increased resistance of water isolates to lower levels of hydrogen peroxide. *Campylobacter* water isolates are naturally present in an environment containing hydrogen peroxide. Aside from human activities leading to the addition of compounds such as hydrogen peroxide into lakes, rivers, seas and oceans, there is a natural contribution, i.e., ozone combined with falling rainwater can produce hydrogen peroxide in levels high enough to be detected (98, 101, 158). Therefore, *Campylobacter* strains resistant to oxygen in water reservoirs would be more likely to survive in that environment.

CGF results for strains organized by source (Table B3) identified three genes (*Cj0264c*, *Cj0177*, *Cj0755c*), which might be important for sensitivity to hydrogen peroxide. In this study, the *Cj0264c* gene was present in 22 (24 total) clinical strains when compared to 13 (24 total) animal and 8 (19 total) water strains. Cj0264c, a molybdopterin containing oxidoreductase reduces trimethylamine-N-oxide (TMAO) and dimethylsulfoxide (DMSO) (4). In the electron transport chain, TMAO is reduced to TMA after it interacts with cytochrome C subunits and accepts two electrons (48). The oxidation of trimethylamine (TMA) with hydrogen peroxide produces TMAO and water (111). Trimethylamine serves as an electron acceptor for oxidative phosphorylation in Enterobacteriaceae and in *Campylobacter* spp. anaerobic environments support the respiration of Enterobacteriaceae in the presence of TMAO and DMSO but in *C. jejun*i, increased growth rates are observed under microaerophilic conditions as compared to anaerobic environments (4, 136). In the presence of hydrogen peroxide, TMA is converted to TMAO but Cj0264c

oxidoreductase converts TMAO back to TMA, which is required for respiration and increased growth. The presence of the *Cj0264c* gene in most of the clinical strains could explain the decreased sensitivity of these isolates to hydrogen peroxide when compared to animal strains (Figure 7B). For water strains obtained from freshwater sources, the concentration of TMAO in freshwater fish was negligible (4). Water strains *Campylobacter* in this study could have lost the *Cj0264c* gene because it was not required, or other mechanisms were found to deal with the stress. It is also possible that in the aerobic freshwater environments, the water *Campylobacter* strains might have used other compounds for respiration.

Iron, an important component of respiration and other metabolic processes in *C. jejuni*, is important for colonization of the human host although it could cause oxidative stress (61) (94)(114). There are several iron uptake systems in *C. jejuni* (94). The iron transport protein Cj0177, part of the Cj0173c-Cj0178 iron uptake system was present in 17 (24 total) clinical, 12 (24 total) animal, and 7 (19 total) water isolates, as determined by CGF experiments. This system is responsible for uptake of iron that is bound to the human ferri-lactoferrin siderophore (94). In presence of hydrogen peroxide, ferrous iron can be oxidized via the Fenton reaction to produce ferric iron and a hydroxyl radical, a reactive oxygen species that could damage the host cell (113, 114). However, iron metabolism is regulated by the cell and is important for several bacterial functions. The *Cj0177* gene, being part of an iron uptake system, could have contributed to the reduced sensitivity of clinical strains to higher levels of hydrogen peroxide but further investigation is required to determine this relationship.

Another iron-acquisition systems in *C. jejuni* is the *cfrA* (*Cj0755*) and CeuBCDE uptake system (94). The ferric enterobactin uptake receptor *cfrA* interacts with ferri-enterochelin to uptake iron and this receptor is bound to the inner membrane ABC transporter system CeuBCDE (94, 114, 167). Isolates of severe human campylobacteriosis cases were commonly found to lack the *cfrA* gene (167). In the avian host, the loss of *cfrA* mutant decreased colonization (94, 114). CGF results showed that only 9 (24 total) clinical strains carried this gene, as compared to 15 (24 total) animal strains and 6 (19 total) water isolates. It was unclear if clinical strains were obtained from severe cases of human campylobacteriosis, as the information was not available. The low incidence of the *cfrA* gene in water strains as determined by CGF, indicates that these isolates may contain certain virulence genes that would increase their pathogenicity in the human host. The presence and absence of genes that play a role in the virulence of *C. jejuni* in humans was recently compared. Identifying differences in these genes between strains from varied sources and comparative genomic fingerprints, could explain the differences in pathogenicity of these isolates.

The three genes (*Cj0264c*, *Cj0177*, *Cj0755c*) were identified using CGF however it would be interesting to compare the presence of *katA* in strains from different sources and clusters. The *katA* gene encodes a catalase regulated by Fur, a ferric uptake regulator, and is important for resistance to hydrogen peroxide (114). For future experiments, all strains could be tested for their sensitivity to other compounds related to oxidative stress, including cumene hydroperoxide and menadione (113).

Assays using Caco-2 cells showed that clinical strains were more adherent and invasive than animal strains (Figure 8). A higher invasion potential of human clinical strains as compared to animal strains was observed in a study comparing 74 chicken and 39 human strains (33). It was determined that the hyperinvasive group of strains contained mostly human isolates (33). Also, chicken isolates were found to be less virulent *in vitro* than clinical strains, because only human isolates from patients suffering from invasive and watery diarrhoea produced cytotoxins (125).

Water strains had the largest distribution of invasive cells counts for isolates organized by cluster (Figure 8C) and by source (Figure 8D). As strains from clinical sources and clinical and animal clusters were both significantly more invasive than isolates from animal clusters and sources, higher invasion cell counts of clinical and animal clusters were mostly due to the clinical strains. The animal source group included all animal strains studied, including those that clustered with the clinical strains. The increased range for invasion of animal isolates organized by source rather than by cluster, showed that there was some contribution of the animal strains to the significantly higher invasion rate of isolates in the clinical and animal cluster. The adherence and invasion potential of the animal strains was similar to a previous study of 43 *C. jejuni* and *C. coli* animal isolates from chicken, turkey, pork and beef that had different adherence and invasion abilities for T84 human colonic epithelial cells (169). For the clinical strains, the majority of isolates in the clinical source group showed similar adherence indicating that genetic relatedness may play a role in adherence for clinical strains, but variability was high for invasiveness. In a study

comparing clinical, food (meat) and live animal 31 (16 *C.* coli and 15 *C. jejuni*) isolates from Mexico, adherence and invasion rates were variable (86).

The adherence (Figure 8B) and invasion (Figure 8D) of *C. jejuni* clinical isolates was significantly greater (Mann-Whitney test; $p<0.05$) as compared to the animal strains, while the water strains had a large distribution. To identify the potential effects that genetic relatedness of the clinical strains could have on adhesion and invasion of animal and water strains, the top 10 highest values of adhesion and invasion for each isolate source were compared for both assays (Table B4). Most of the strains with increased adherence and invasion to Caco-2 human cells were genetically related by CGF to the clinical strains. However, this relatedness was always necessary, as isolates from clusters of only animal or only water isolates were also found to adhere and invade in high numbers. In every source group, some strains were found to be among the most adherent and invasive. However, the majority of strains that were highly invasive were not the most adherent. Some clinical, animal and water strains had increased adherence and invasion to Caco-2 cells, indicating pathogenic potential of strains from varied sources in the human host.

Strains isolated from patients with severe diarrhoeal disease would be expected to have a higher adhesion and invasion potential when compared to isolates from individuals with non-inflammatory symptoms, from chickens or from environmental sources (78, 79, 87). The virulence properties of the clinical isolates were not assessed in this study based on patient symptoms, as the information was not available. However, clinical strains from different genetic clusters displayed

variability in their ability to adhere and invade Caco-2 cells. For example, strain CGY_HR_071 from cluster 179 was a high invader while isolate CGY_HR_074 from cluster 516 was a low invader. As water and animals are known vehicles for *Campylobacter* infection, at least a subset of the strains isolated from these sources would be expected to cause human infection.

Comparative genomic fingerprinting results were compared for strains sorted by cluster (Table B2) and by source (Table B3). Genes *Cj0177* and *Cj0486* were linked to adherence and invasion in *C. jejuni*. *Cj0177* was more prevalent in clinical isolates (17 of 24) than animal (12 of 24) and water (7 of 19) strains. All isolates in the clinical and animal cluster combined (15 total) had this gene but strains in the water clusters (2 of 12) and those in the animal clusters (4 of 11) had very low numbers. This gene encodes for an iron transport protein involved in the iron uptake system *Cj0173c*-*Cj0178* as previously discussed. The presence of iron encourages host colonization and iron limitation is a host defense mechanism against bacteria (61). In order for strains to be recovered from human patients, they have to overcome the immune system and colonize the host. Clinical strains would have benefited from the expression of *Cj0177* and *Cj0178*. The higher number of clinical isolates carrying the *Cj0177* and possibly *Cj0178* could be the reason for the increased adherence that was observed for these strains.

The fucose permease Cj0486 (*fucP*) is part of a genomic island (Cj0480c-Cj0490) important for L-fucose metabolism and is responsible for the transport of this sugar in *C. jejuni* (33, 141) (38, 116). *Cj0486* mutants have significant decreased colonization in the piglet model of human disease, but not in the

commensal chicken model, when compared to the wild-type strain (141). Expression of this gene is a contributing factor for *C. jejuni* pathogenesis in humans because it promotes survival of this bacterium in the host (33, 141). The host releases L-fucose when *C. jejuni* colonizes the intestine and some strains have the ability to uptake and metabolize this sugar through a unique pathway (141)(167). Fucose is a chemoattractant and a carbone source promoting growth and contributing to colonization and invasion of some *C. jejuni* strains in the host (141). When comparing clinical, animal and water isolates, the clinical strains showed greater adherence to the Caco-2 human cell line, which corroborated with previous findings (78). However, the *Cj0486* gene was present in 9 (of 24) clinical strains, in 18 (of 24) animal isolates and in 4 (of 19) water isolates (Table B3). When separated in clusters, this gene was present in all isolates from clinical and animal clusters (15 total), in 2 isolates belonging to water clusters (12 total) and in all strains from only animal clusters (11 total) (Table B2). This gene was present in most of the animal strains and in clinical isolates genetically related to them. This is similar to results obtained by Zautner *et al.* (167) that showed an increased ability to metabolize L-fucose in livestock-adapted strains. It was absent in most of the clinical strains that invaded the human host. Among the 10 most adherent and invasive strains (Table B4), 4 of the most adherent (BCC-67, CGY_HR_072, CGY_HR_098, CHR_151) and 3 of the most invasive (BCC-67, CGY_HR_082, CGY_HR_098) isolates carried the *Cj0486c* gene. Previously, both high and low invasive *C. jejuni* strains were found to carry this gene (33). For future studies, it would be of interest to determine the colonization and invasion of strains from varied sources in the piglet and chicken models. As *Cj0486* was absent in most of the clinical strains studied, identifying

genes that are up-regulated and down-regulated could explain the mechanism used by *C. jejuni* to colonize the host. Pathways can be further elaborated for strains from varied sources to potential identify other factors that may contribute to invasion in *C. jejuni*, possibly genes that contribute to the increased invasion of some strains in presence of *Cj0486*. This could also give insight into *C. jejuni* defense mechanisms in the host and the identification of potential target genes for human as well as chicken vaccines.

When combining phenotypic results into a hierarchical clustering map (Figure 9) to identify similarities between strains from varied sources and clusters, clinical and animal isolates had greater similarity to each other when compared to water isolates. This is observed for low and high values for each phenotypic assay. This was expected as based CGF results (Figure 1) in this study, the majority of water strains were in environmental only clusters. This indicates that the phenotype varied according to clusters and sources. This is similar to a study by Read *et al.* comparing genotypic and phenotypic results for 108 *Campylobacter* strains and also considering strain source (127). They determined that strains from the same MLST clonal complex had differences in phenotypic characteristics (127). However, when separating strains in the same clonal complex based on source group, phenotypic tendencies were significantly similar between strains (127). This further validates the importance of identifying differences between strains in our study based on CGF clusters and particularly based on source groups.

Biofilm formation in *Campylobacter* is a relatively new aspect that is being studied for these bacteria and results of several studies can be contradictory.

However, similarities in biofilm formation between strains form the varied sources have been demonstrated previously. When comparing the clinical isolates M129 to the animal isolate S2B, Reeser *et al*. (128) observed similar biofilm forming potential. Furthermore, a study of only clinical strains showed differences in biofilm formation that may be linked to variations in hydrophobicity of the strain (71). The importance of strain source and cluster for biofilm formation in *Campylobacter* requires further investigation.

Several important metabolic pathways and genes have been studied with the goal of understanding the relationship between oxidative stress and *Campylobacter* (Atack_2009). In presence of hydrogen peroxide, an oxidant, oxidative stress defense genes are expressed in *Campylobacter* (113) and several studies have looked at differences between mutants and the respective wild-type strain. However, a clustering has not been studied to our knowledge.

The hierarchical map (Figure 9) also illustrated that regardless of the source or CGF cluster, phenotype results for motility, adhesion and invasion clustered together. This is to be expected since motility is important for both adhesion and invasion of *Campylobacter* in host cells (34, 79). These three properties are important for *Campylobacter* colonization and infection in the host (14, 75).

4.2.2 Microarray analysis

A pan-genome *C. jejuni* DNA microarray was used to determine differences in genes expressed (RNA channel) or present (DNA channel) between clinical, animal and water sources. Analysis was done for 14 animal, 12 water and 13 clinical strains using microarrays.

Strains from clinical, animal and water sources were compared by microarray for the presence of genes (Figure 10A) and lists of genes were populated (Tables C1 to C3). In the “DNA: C vs W” comparison (Figure 10A, Blue circle, Table C1) a higher number of clinical strains had genes possibly associated with resistance to hydrogen peroxide (*Cj0045c* and *Cj1013c*) and for motility (*Cj0336c* and *Cj1408*), as well as the cytolethal-distending toxin subunit A encoded by the *Cj0079c* gene (169) and the putative MCP-domain signal transduction protein Cj0951c important for invasion (166). Among the genes found (Table C2) that were more likely to be associated with animal isolates compared to water isolates (Figure 10A, Yellow circle), were those associated with sensitivity to hydrogen peroxide (*Cj0045c*, *Cj1224*, *Cj1397*, *Cj1569c* (136), *Cj1616*) and motility (*Cj1318*) as well as the *Cj1221* gene encoding a chaperonin GroEL linked to biofilm formation (74), the pathogenicity markers *Cj1351* and *Cj1365c* encoding a phospholipase A and serine protease, respectively (167, 169), and the *Cj0079c* gene encoding the cytolethal-distending toxin (169). When compared to clinical strains, animal strains were significantly more likely to encode a number of genes (Figure 10A, Green circle, Table C3). Genes over-represented in animal strains included the *Cj1569c* (136), *Cj0241c*, *Cj0271*, *Cj1397* and *Cj1430c* genes important for sensitivity to hydrogen peroxide, the *Cj0526c* and *Cj0527c* genes for, motility as well as the *Cj1221* gene for biofilm formation (74).

This list of genes present in animal strains compared to water or clinical strains (Figure 10A) groups included the *Cj1221* gene, which was previously found to be overexpressed in biofilm-grown cells (74). Biofilm results however showed that

the water strains were better biofilm formers than the animal strains (Figure 6). There was also the *Cj1569c* gene encoding the NADH dehydrogenase I chain K that was linked to sensitivity of *C. jejuni* to hydrogen peroxide (136). This corroborates phenotypic data indicating an increased sensitivity to hydrogen peroxide for animal strains (Figure 7).

Gene content of *C. jejuni* integrated elements genes was also presented in a hierarchal clustering map (Figure C1). Genes from each insertion clustered together indicating similarities in levels of presence between them. When comparing presence of phage-associated genes in clinical, animal and water isolates, a higher proportion of animal (79%) strains included genes belonging to the *Campylobacter* Mu-Like Phage 1 insertion compared to 62% of clinical and 42% of water isolates (Table C4). A previous study comparing 67 clinical and animal isolates determined that 20% of clinical and 40% of animal strains contained CJIE1 genes (115). A greater percentage of animal strains included genes belonging to all four insertions when compared to clinical and water isolates. This difference in the presence of insertions may be due the transmission of strains from livestock to environmental and clinical sources. *Campylobacter* strains are often transmitted from animal sources to water and clinical sources (21), which could cause changes in genomic content and possibly leading to the loss of phage genes. Sixty-two percent of strains from clinical, animal and water sources included genes from CJIE1 indicating a somewhat frequent presence of the *Campylobacter* Mu-like prophage in isolates from varied sources. A higher number of phage-related genes were present from CJIE2 when compared to CJIE4 indicating that genes in CJIE2 may be more

common in *C. jejuni*. Clinical strains had the smallest proportion of genes present from CJIE2 and CJIE4 when compared to animal and water isolates. Overall, these results showed that the presence of phage and phage-related genes was variable among strains from varied sources, with higher levels in animal strains.

For differential expression (Figure 10B), list of genes were populated based on whether a gene was both present and transcribed at any level (Tables C5 to C7). When comparing "RNA: C vs W" group (Blue circle), the list (Table C5) included two genes, *Cj0264c* (136) and *Cj0436*, more often found in the 14 water strains, and which may play a role in sensitivity to hydrogen peroxide. Among the genes that were more often associated with the clinical strains, three are important for motility (*Cj0527c*, *Cj0528c* and *neuA1*) and one associated with sensitivity to hydrogen peroxide (*Cj1020c*). The significant number of water strains expressing *Cj0264c* could explain the decreased sensitivity of water strains to 0.10 µmoles hydrogen peroxide (Figure 7B).

Among the genes (Table C6) more likely to be present and expressed in animal strains compared to water (Figure 10B, Yellow circle), 11 of them (*Cj1013c*, *Cj0081*, *Cj1516*, *Cj1509c*, *Cj1398*, *Cj1658*, *Cj0436*, *Cj0369c*, *Cj0174c*, *Cj0265c* (136) and *folD*) may play a role in sensitivity to hydrogen peroxide. Four genes linked to virulence in *C. jejuni* were found including those encoding the fucose permase Cj0486, the cytolethal-distending toxin A Cj0079c (169), and the flagellin *O*-glycosylation genes (Cj1324c and Cj1365c).

In the list of genes (Table C7) more likely to be actively transcribed in animal strains compared to clinical strains (Figure 10B, Green circle) group of differentially

expressed genes, nine genes are thought to play a role in sensitivity to hydrogen peroxide (*Cj0081*, *Cj1013c*, *Cj1411c*, *Cj0241c*, *Cj1020c*, *napB*, *fliY*, *Cj0045c*, *folD*), and 7 are important for motility (*Cj0319*, *Cj0527c*, *Cj0337c, Cj0887c*, *Cj0687c*, *Cj0528c*, *Cj0041c*). The *Cj0882c* genes encoding the flagellar biosynthesis protein FlhA has been linked to biofilm formation (74) while *Cj0486* and *Cj0914c* (169) encoding the fucose permase and CiaB genes, respectively, are important for invasion. The *Cj0486* gene is part of a genomic island (*Cj0480c*-*Cj0490*) that is significantly up-regulated in presence of L-fucose, a carbon source contributing to invasion of some *C. jejuni* strains in the host (141). The expression of CiaB is an indicator of increased invasion potential, which would lead to the conclusion that animal strains may be more invasive than clinical strains. However, the opposite was observed and clinical strains were significantly more invasive to Caco-2 cells than animal strains (Figure 9D). The flagellin *O*-glycosylation gene *Cj1324c* (167) linked to virulence was also among the genes with higher expression.

Fifty-six genes were common between the “RNA: A vs W” and “RNA: A vs C” groups (Figure 10B). The two groups contained genes that were more often expressed in animal strains so this combination identifies genes that were similarly more expressed in animal strains. Genes more often found in animal strains included those may be important for sensitivity to hydrogen peroxide (*Cj0081*, *Cj0436*, *Cj1013c*, *folD*), the fucose permase *Cj0486* involved in colonization (141), and the *Cj1324* gene encoding the flagellin *O*-glycosylation (166). The *Cj0081*-*Cj0082* genes encode a cytochrome bd oxidase (cydAB) involved in respiration and that was up-regulated in iron rich conditions (12, 108). The pyridoxamine 5’-

phosphate oxidase (Cj0436) catalyses the oxidation of pyridoxamine 5'-phosphate producing ammonia and hydrogen peroxide, which was previously studied in *E. coli* (168). This gene may contribute to oxidative stress in *Campylobacter* but further investigation is required. The cytochrome C biogenesis protein encoded by the *Cj1013c* gene, was found down-regulated n the presence of ciprofloxacin (54)). *Rhodobacter capsulatus* had differential expression of genes in the operon encoding Cytochrome C biogenesis protein based on the presence of oxygen (42). Future studies for this protein in *Campylobacter* may determine a role for the *Cj1013c* gene in oxygen metabolism and possibly, oxidative stress. The flagellin *O*-glycosylation locus (*Cj1321-Cj1326*) was associated with strains having decreased pathogenicity, as they were found in animals and the environment (15, 167).

There were also 15 genes similarly expressed in "RNA: C vs W" and "RNA: A vs C" groups (Figure 10B). These included the *Cj0527c* and *Cj0528c* genes, respectively, encoding the flagella basal body rod proteins FlgC and FlgB, as well as the *Cj1020c* gene encoding a putative cytochrome C. In the presence of hydrogen peroxide, cytochrome c was degraded (37). The *Cj1020c* gene was expressed in more clinical strains than water strains, and in more animal strains than clinical strains. This pattern is similar to results for sensitivity to hydrogen peroxide, as animal strains were the most sensitive followed by clinical and then water isolates. It appears that the increased expression of this cytochrome C may lead to increased sensitivity of animal strains to hydrogen peroxide.

Genes with significantly different levels of relative transcript abundance (RNA/DNA) among groups were identified using a Student's T-test (Table C8 and

C9). While there were 2 genes having higher expression values in clinical strains when compared to water isolates, animal strains had 17 genes with significantly higher abundance in transcripts than clinical isolates (Table C8). The *Cj0480c* gene encoding a transcriptional regulator, part of the genomic island (*Cj0480c*-*Cj0490*) was previously found to be significantly up-regulated in the presence of L-fucose (141). Fucose may be important for early stages of colonization in the chick model (141). The other genes in this genomic island did not have significantly higher transcript levels possibly indicating differences in regulation. Factors influencing the up-regulation of the *Cj0480c* gene in animal strains when compared to clinical strains are still unclear and require further investigation. The *Cj1501* gene encodes a hypothetical protein involved in the selenium-dependent formation of the formate dehydrogenase (137). This latter protein may be important for the clearlng of formate produced due to anerobic respiration in the chicken cecum (137). The importance of oxygen for the clearing of formate from the host cell is unsure however oxidization of formate is important to remove it from the cell and oxidative stress is not clear and requires further investigation. Lastly, the *Cj1365c* gene encoding a secreted serine protease may be important for virulence in the host (167). The absence of this gene was common among strains that caused human campylobacteriosis, bloody diarrhea and hospitalization (167). The observation that this gene is more expressed in animal stains relative to clinical corroborates with the literature. Also, the *Cj1365c* had higher expression in animal strains when compared to water isolates. This could also explain certain phenotypic results where some water strains demonstrated higher virulence properties when compared to animal strains.

There were 58 genes found to have significantly higher abundance of transcripts in animal strains when compared to water isolates (Table C8). Genes encoding the transcriptional regulator Cj0480c, the short chain dehydrogenase Cj0485, the amidohydrolase Cj0487, and the aldehyde dehydrogenase C-terminus Cj0490, are all part of the genomic island (*Cj0480c-Cj0490*) that is up-regulated in presence of L-fucose and mucin (141). With most of the genes in this genomic having higher expression in animal strains relative to water isolates, this could explain that the presence of fucose is important for animal isolates possibly for growth or colonization in the host. Further investigation is required to determine the importance of fucose for water isolates to better understand the decreased expression of these genes in their genomes.

The *Cj1417c* gene encodes an amidotransferase that is part of the capular polysaccharide locus (*Cj1417c-Cj1442c*). According to a previous study by Pope *et al.* (122), strains carrying some of these genes seem to have a competitive advantage when colonizing the chicken intestine. Strains that lacked the *Cj1417c*, *Cj1420c*, and *Cj1442c* appeared to have decreasd virulence and colonization potential in the chicken gut (122). In this study, animal strains had a higher expression level of the *Cj1417c* gene possibly because this promoted colonization of animal strains in the chicken gut but did not influence survival of water isolates in the environment. Also, an MCP-domain signal transduction protein Tlp7 (Cj0951c) was previously found to be involved in invasion of *Campylobacter* strains to the Caco-2 intestinal cell lines (148). The *cdtA* (Cj0079c) gene encoding a cytolethal distending toxin A (Cj0079c) had increased expression in animal strains when compared to

clinical isoaltes. Taken together, the higher expression of *Cj0951c* and *cdtA* could indicate incrased potential for pathogenesis of *Campylobacter* animal isolates in the human host when compared to water isolates however further investigation is required.

Several genes possibly involved in oxidative stress resistance were found up-regulated in animal strains when compared to water isolates (Table C8). The *Cj1371* gene encoding a periplasmic protein was found to increase resistance of *C. jejuni* strains to paraquat, an oxidizing agent (45). Also, the Cj0637c gene encoding a methionine sulfoxide reductase A was show to increase resistance to hydrogen peroxide, organic peroxide and superoxide that supports the involvement of Cj0637c in oxidative stress defence (3). Cj0864, hypothetical protein oxygen tolerase was previously correlated with increase oxygen tolerance of *C. jejuni* isolates (73). These observations require further investigation to understand the role these genes would have on oxidative stress resistance of strains when colonizing the animal host and in water environments. Significant differences in sensitivity to hydrogen peroxide were not observed when comparing animal strains to isolates in the water source group (Figure 7B).

The *Cj0715* and *Cj0716* had higher expression in animal strain when compared to clinical strains that in turn had greater abundance in transcript levels than water isolates (Table C8). However, *Cj0038c*, *Cj1365c*, *Cj1388*, *Cj1404* genes were more highly expressed in animal strains when compared to isolates in the clinical as well as the water source groups (Table C8).

When comparing strains from all source groups, 8 genes were down-regulated (Table C9). The gltB (*Cj0007*) gene had lower relative abundance in transcripts in animal strains when compared to clinical strains that had lower expression than water strains. Several genes encoding flagellar components had lower expression in animal strains when compared to water isolates. These genes include flagellar hook protein FlgE (*Cj1729c*), the flagelllar basal body rod modification protein FlgD (*Cj0042*) and, flagellar basal-body rod protein FlgG2 (*Cj0697*). These genes may be important for motility in *C. jejuni*; however, when comparing animal and water sources in this study, there were no significant differences observed (Figure 5C). The *Cj0012c* gene encoding a non-heme iron protein had decreased expression in response to paraquat, an oxidizing agent indicating a possible role of this gene in oxidative stress defence (45). This was also observed for the *Cj1357c* gene encoding a periplasmic cytochrome C involved in nitrosative stress (119). Further investigation is required to understand the relationship these genes would have on virulence in *C. jejuni*.

Clustering relative expression of genes (RNA/DNA) was represented in a hierarchical clustering map (Figure C2), where replicate experiments clustered together indicated reliability of results. This is expected as these are biological replicates of the same strain and are expected to have similar gene expression values. Also, clinical and water strains seem to cluster together while animal strains are separated, similar to the observations of the whole genome microarray analysis. There might be some variations observed due to experiment procedures particularly RNA extraction, the type of dye used and the method of labeling cDNA sequences

(18). When tested on the whole genome *Campylobacter* microarray, the majority of animal strains clustered together indicating similarities in gene expression profiles between them. Results of clinical and water isolates were variable although there were clusters of strains from each source. There have been publications of comparative genomic analysis for strains from varied sources (15, 59) but gene expression population studies comparing strains from clinical, animal and water sources were not found.

To achieve a more detailed analysis on the presence and expression of genes in strains from varied sources, the isolates could be sequenced using the RNAseq method (104). This method would determine the transcript profiles of each strain and would also provide more information on genetic content.

4.3 *C. jejuni* mutant strain analysis

Two *C. jejuni* mutant strains, Δ*Cj0075c* and Δ*Cj1373* were successfully constructed and tested for motility and sensitivity to hydrogen peroxide. Genes *Cj0075c* and *Cj1373* did not directly influence *C. jejuni* motility. There are no publications describing the importance of *Cj0075c* and *Cj1373* genes for motility in *C. jejuni*. Being an integral membrane protein, *Cj1373* expression could influence genes involved in *C. jejuni* motility, but this would require further investigation. As part of the non-flavin iron-sulfur oxidoreductase (Cj0075c-73c) responsible for intracellular oxidation of L-lactate to pyruvate, the gene *Cj0075c* plays an important role in the growth of *C. jejuni* on L-lactate, alongside the Cj1585c oxidoreductase (150). As these two genes work together, perhaps motility was not affected with the presence of the functional *Cj1585c* gene. Future experiments, could include

performing a motility assay on the double mutant to determine the importance of these two genes for motility in *C. jejuni*.

The importance of *Cj1373* for the sensitivity of *C. jejuni* to hydrogen peroxide and oxidative stress has not been observed previously. The oxidization of L-lactate into pyruvate by the Cj0075c-73c complex provides electrons for the electron transport chain to reduce oxygen into water (150). With the inactivation of the *Cj0075c* and subsequent blocking of an oxidoreductase complex, there were increased oxygen levels inside the microaerophilic *C. jejuni*. In turn, this could have caused increased sensitivity to higher concentration of hydrogen peroxide.

Future experiments with these mutant strains could include adhesion and invasion assays with Caco-2 human epithelial cells to determine the importance of these two genes for virulence in the human host. Transcript profiling could also be performed to identify up- and down-regulated genes in the absence of *Cj0075c* and *Cj1373*.

5: CONCLUSION

Genetic and phenotypic traits of various *C. jejuni* strains from clinical, animal and water sources were compared. Based on results from adhesion, invasion and sensitivity to hydrogen peroxide, clinical strains appear to have more features linked to infectivity. At higher levels of hydrogen peroxide, clinical strains were significantly less sensitive than the animal strains. In addition, clinical strains were significantly more adherent and more invasive than animal isolates. As for the water strains, a large distribution of phenotypes was observed for most experiments. This indicates that a subset of water strains could be of public health significance. Identifying the genetic and phenotypic markers that could differentiate water and animal strains based on pathogenicity, would be an asset to controlling the spread of *Campylobacter* in water treatment plants and farms.

As a high proportion of strains from various sources contained phage or phage-associated genes, understanding the influence of these insertions into *Campylobacter* is important. When comparing phage-containing clinical *C. jejuni* isolates to a strain devoid of phage, decreased motility was observed for the latter strain. However, these results were inconclusive, as only four strains were compared. To assess the influence of prophage on *Campylobacter*, it would be interesting to compare phage-containing clinical isolates with those strains that carry the Mu-Like prophage in animals and in the environment. Determining if the prophage truly increases virulence in *C. jejuni* may help to explain survival in varying

environments and pathogenicity in humans.

Microarray technologies used in this study provided information about the genes expressed and genes present in each strain. However, the information was restricted to the genes printed on the microarray slides. Also, as sample nucleic acids bind to short DNA fragments on the slides, derived from only a single strain, important genes may have been missed in this analysis. To address this, RNA sequencing experiments are underway to more accurately determine the number of transcripts for each gene. All of the strains in this study, including the phage-containing isolates and the mutant strains, will be further analyzed using RNA sequencing.

A number of genetic markers associated with *C. jejuni* strains isolated from different sources were identified. To determine the role of these genes in infectivity and survival of the organism, it will be necessary to perform mutational analysis on a number of these markers. In this study, two mutants were assessed; however, the effects of the mutation could not be identified by phenotype experiments alone. RNA sequencing of these mutants may provide clues about the effect of the mutation on the organism. Mutational analysis of a large number of genes will be necessary to elucidate key pathways that may impact growth and survival in different environments.

To get a more accurate depiction of the role the source of strains could play on their pathogenicity, a greater number of isolates is required from each source. These strains should be selected based on phenotypic traits for motility, biofilm

formation, adhesion, invasion and sensitivity to hydrogen peroxide. Agglutination and cytokine release studies could also provide valuable information on infectivity and survival. Comparing gene expression of virulent strains to those appearing to be less virulent, might identify genetic markers specific to more pathogenic strains. For this study to be meaningful, a minimum of 100 strains from each source group (clinical, animal, water) would be required to allow the formulation of broadly applicable conclusions. With a higher number of strains, a pattern of genetic and phenotypic traits would be more evident than when comparing a small group of isolates. For less virulent strains, understanding their role in the pathogenicity of *C. jejuni* would also be important to determine if their role in the transmission of the infection. Using animal models, in which the virulent and less virulent strains serve as commensals, would be the next step. Comparing transcript profiles of virulent and less virulent isolates when exposed to animal or human cells might provide insight into their pathogenicity in humans. Recently, over 130 full genome sequences of *Campylobacter* strains from varied sources were completed in the lab. Sequence analysis using bioinformatics tools is underway to more specifically identify genetic markers associated with each source.

Virulent strains of *C. jejuni* were found among strains from clinical, animal and water sources, indicating that the risk of human infection was present at every step of the food chain. Identifying virulence factors could lead to a better control of this bacterium, decrease the number of infections every year and minimize illness costs associated with *Campylobacter*.

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CONTRIBUTIONS OF COLLABORATORS

The clinical, animal and water isolates and microarray slides were provided by Dr. Eduardo Taboada from the Laboratory for Foodborne Zoonoses of Public Health Agency of Canada, Lethbridge, Alberta, Canada. The phage-containing *C. jejuni* isolates and the control strain were provided by Dr. Clifford Clark from the National Microbiology Laboratory of Public Health Agency of Canada, Winnipeg, Manitoba. The *E. coli* strains used for the mutant construction were provided by Dr Alain Stintzi from the Ottawa Institute of Systems Biology of the University of Ottawa.

Comparative Genomic Fingerprinting results and the phylogenetic tree of strains from varied sources were provided by Steven Mutschall from the Laboratory for Foodborne Zoonoses of Public Health Agency of Canada, Lethbridge, Alberta, Canada. The “RNA vs gDNA” microarray protocol was designed with the help of Nathalie Corneau from the Bureau of Microbial Hazards, Health Canada, Ottawa, ON, Canada.

Statistical analysis of microarray data and real-time PCR was performed by Catherine Carrillo from the Bureau of Microbial Hazards of Health Canada, Ottawa, ON, Canada.

APPENDICES

Appendix A: Tables and figures for methods

Table A1. *Campylobacter* isolates from animal, clinical and environmental sources. Strains from varied sources were isolated from Alberta, British Columbia, and New Brunswick. *Campylobacter* isolates of animal origin were isolated from avian and bovine sources. A majority of the water isolates were collected from the Old Man River Basin (OMRB) in Alberta.

Strain	Description	Source	Species
06_2866	Avian (Chicken): Chicken Breast; C-EnterNet	Animal	*C. jejuni*
CI-0187	Avian (Chicken): Faecal; Alberta	Animal	*C. jejuni*
CI-0225	Avian (Chicken): Faecal; Alberta	Animal	*C. jejuni*
CI-0233	Avian (Chicken): Faecal; Alberta	Animal	*C. jejuni*
CI-0236	Avian (Chicken): Faecal; Alberta	Animal	*C. jejuni*
CI-0237	Avian (Chicken): Faecal; Alberta	Animal	*C. jejuni*
CI-0334	Bovine (Cow): Faecal; Alberta	Animal	*C. jejuni*
CI-0521	Avian (Chicken): Faecal; Alberta	Animal	*C. jejuni*
CI-0522	Avian (Chicken): Faecal; Alberta	Animal	*C. jejuni*
CI-0546	Avian (Chicken): Faecal; Alberta	Animal	*C. jejuni*
CI-0554	Avian (Chicken): Faecal; Alberta	Animal	*C. jejuni*
CI-0558	Avian (Chicken): Faecal; Alberta	Animal	*C. jejuni*
CI-0676	Avian (Chicken): Faecal; Alberta	Animal	*C. jejuni*
CI-0684	Avian (Chicken): Faecal; Alberta	Animal	*C. jejuni*
CI-0986	Bovine (Cow): Faecal; Alberta	Animal	*C. jejuni*
CI-0987	Bovine (Cow): Faecal; Alberta	Animal	*C. jejuni*
CI-1671	Avian (Chicken): Faecal; Alberta	Animal	*C. coli*
CI-1674	Avian (Chicken): Faecal; Alberta	Animal	*C. jejuni*
CI-1678	Avian (Chicken): Faecal; Alberta	Animal	*C. coli*
CI-1689	Avian (Chicken): Faecal; Alberta	Animal	*C. coli*
CI-1969	Avian (Chicken): Faecal; Alberta	Animal	*C. jejuni*
CI-1970	Avian (Chicken): Faecal; Alberta	Animal	*C. jejuni*
CI-2057	Avian (Duck): Faecal; Alberta	Animal	*C. jejuni*
CI-2062	Avian (Chicken): Faecal; Alberta	Animal	*C. jejuni*
07_4268	C-EnterNet	Clinical	*C. jejuni*
08_7017	C-EnterNet	Clinical	*C. jejuni*
BCC-39	British Columbia Clinical and Environmental	Clinical	*C. jejuni*
BCC-62	British Columbia Clinical and Environmental	Clinical	*C. jejuni*
BCC-67	British Columbia Clinical and Environmental	Clinical	*C. jejuni*
BCC-70	British Columbia Clinical and Environmental	Clinical	*C. jejuni*
CGY_HR_015	Calgary Health Region	Clinical	*C. jejuni*
CGY_HR_025	Calgary Health Region	Clinical	*C. jejuni*
CGY_HR_058	Calgary Health Region	Clinical	*C. jejuni*
CGY_HR_071	Calgary Health Region	Clinical	*C. jejuni*
CGY_HR_072	Calgary Health Region	Clinical	*C. jejuni*

CGY_HR_074	Calgary Health Region	Clinical	*C. jejuni*
CGY_HR_082	Calgary Health Region	Clinical	*C. jejuni*
CGY_HR_085	Calgary Health Region	Clinical	*C. jejuni*
CGY_HR_090	Calgary Health Region	Clinical	*C. jejuni*
CGY_HR_098	Calgary Health Region	Clinical	*C. jejuni*
CGY_HR_102	Calgary Health Region	Clinical	*C. jejuni*
CGY_HR_108	Calgary Health Region	Clinical	*C. jejuni*
CGY_HR_109	Calgary Health Region	Clinical	*C. jejuni*
CGY_HR_120	Calgary Health Region	Clinical	*C. jejuni*
CGY_HR_130	Calgary Health Region	Clinical	*C. jejuni*
CGY_HR_208	Calgary Health Region	Clinical	*C. jejuni*
CHR_037	Chinook Health Region	Clinical	*C. jejuni*
CHR_053	Chinook Health Region	Clinical	*C. jejuni*
CHR_151	Chinook Health Region	Clinical	*C. jejuni*
CI-0109	Little Bow River: LBW-04; OMRB (Alberta)	Water	*C. jejuni*
CI-0292	Battersea Drain: B04-4; OMRB (Alberta)	Water	*C. jejuni*
CI-0310	Battersea Drain: B05; OMRB (Alberta)	Water	*C. jejuni*
CI-0475	Little Bow River: LB04-02; OMRB (Alberta)	Water	*Campylobacter*
CI-0532	Little Bow River: LB04-02; OMRB (Alberta)	Water	*C. jejuni*
CI-0571	Little Bow River: LB04-02; OMRB (Alberta)	Water	*C. jejuni*
CI-0578	Little Bow River: LBW-03; OMRB (Alberta)	Water	*C. jejuni*
CI-0599	Grand Falls: WTP Weir #12; New Brunswick	Water	*C. jejuni*
CI-0616	Battersea Drain: B04-4; OMRB (Alberta)	Water	*C. jejuni*
CI-1415	Battersea Drain: AB6; OMRB (Alberta)	Water	*C. jejuni*
CI-1446	Grand Falls: WTP Weir #12; New Brunswick	Water	*C. jejuni*
CI-1814	Salmon River: Salmon Fish Counter (Site 05); British Columbia Clinical and Environmental, British Columbia	Water	*C. jejuni*
CI-2200	Little Bow River: AB7 LBW-01; OMRB (Alberta)	Water	*C. jejuni*
CI-2822	Battersea Drain: AB6; OMRB (Alberta)	Water	*C. jejuni*
CI-2847	Lethbridge: WTP; OMRB (Alberta)	Water	*C. jejuni*
CI-2916	Battersea Drain: AB6; OMRB (Alberta)	Water	*C. jejuni*
CI-2995	Little Bow River: AB7 LBW-01; OMRB (Alberta)	Water	*C. jejuni*
CI-3000	Lethbridge: WTP; OMRB (Alberta)	Water	*C. jejuni*
CI-3039	Lethbridge: WTP; OMRB (Alberta)	Water	*C. jejuni*

Figure A1. Motility and biofilm formation assays. For the motility assay (Figure A1-A), strains were grown for 24 h in microaerophilic conditions before being diluted to an OD_{600} of 0.025 (Step A). A 1 µL volume was stabbed into a 0.4% MHA plate (Step B) and plates were incubated for 48 h (Step C). To determine displacement, the diameter of the bacterial circle produced by the movement of the bacteria, was measured using a ruler (Step D). For the biofilm assay (Figure A1-B), a 24 h bacterial culture was incubated in MH broth for another 24 h (Step A). Cultures were then diluted to an OD_{600} of 0.05 (Step B) and samples were put in 4 wells of three well plates to test biofilm formation after 24, 48 and 72 h (Step C). After every 24 h period, crystal violet was added directly to each well before incubating for 15 minutes at room temperature and washing the well with water (Step D). Plates were left to dry before taking a picture (Step E). DMSO was added to each well to dissolve the crystal violet and absorbance measurements at 570 nm were taken after 24-48 h (Step F).

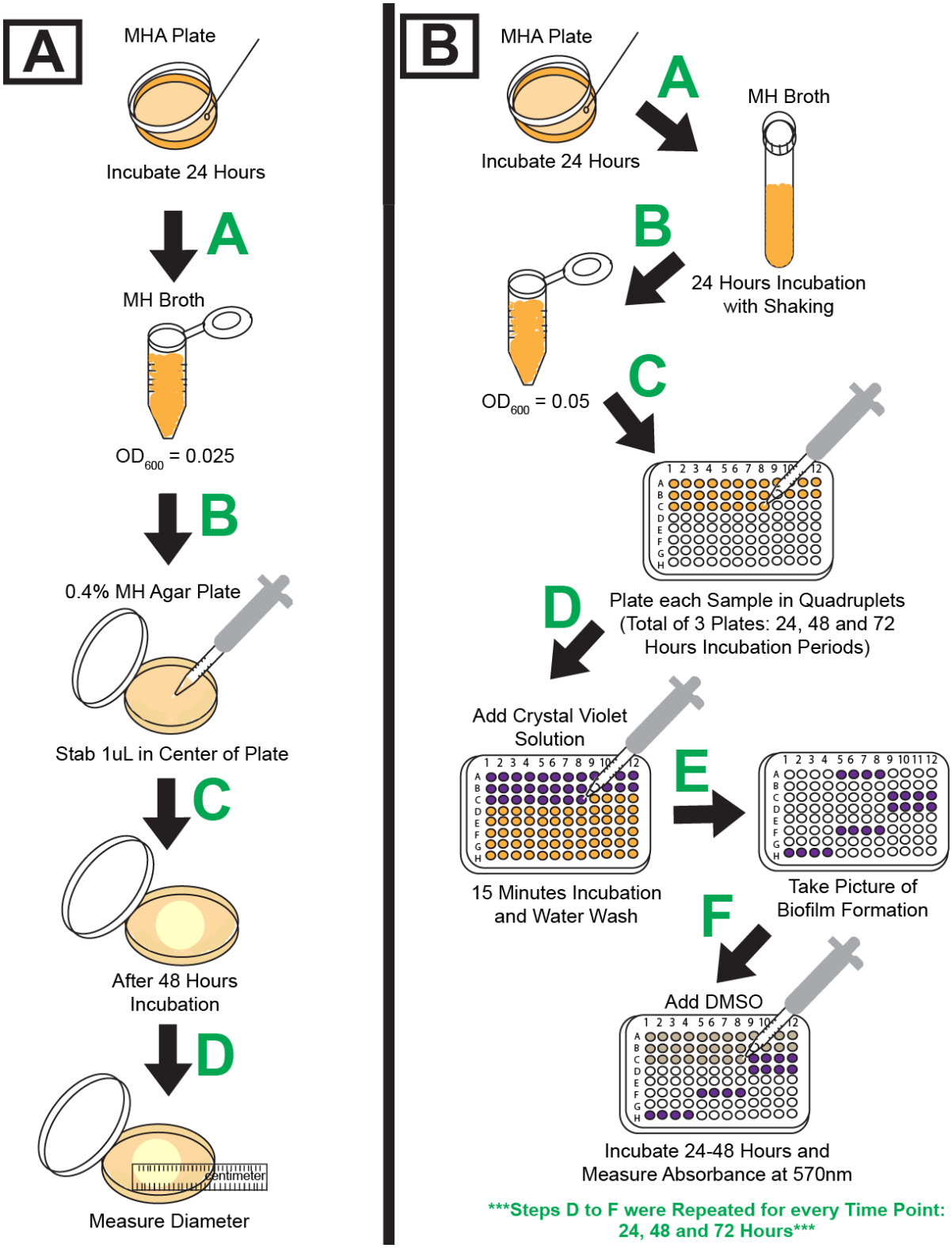

A
MHA Plate
Incubate 24 Hours
A
MH Broth
OD600 = 0.025
B
0.4% MH Agar Plate
Stab 1uL in Center of Plate
C
After 48 Hours Incubation
D
centimeter
Measure Diameter
B
MHA Plate
Incubate 24 Hours
A
MH Broth
B
24 Hours Incubation with Shaking
OD600 = 0.05
C
D
Plate each Sample in Quadruplets (Total of 3 Plates: 24, 48 and 72 Hours Incubation Periods)
Add Crystal Violet Solution
15 Minutes Incubation and Water Wash
E
Take Picture of Biofilm Formation
F
Add DMSO
Incubate 24-48 Hours and Measure Absorbance at 570nm
Steps D to F were Repeated for every Time Point: 24, 48 and 72 Hours

Figure A2. Hydrogen peroxide sensitivity assay. Bacteria grown for 24 h on MHA plates were collected in 1 mL MH Broth and diluted to an OD_{600} of 0.2 (Step A). One hundred microliters was plated to form bacterial lawn on MHA plates and discs with varying amounts of hydrogen peroxide were placed on top of the bacteria (Step B). After 48 h incubation (Step C), the diameter of the zone of inhibition was measured (Step D).

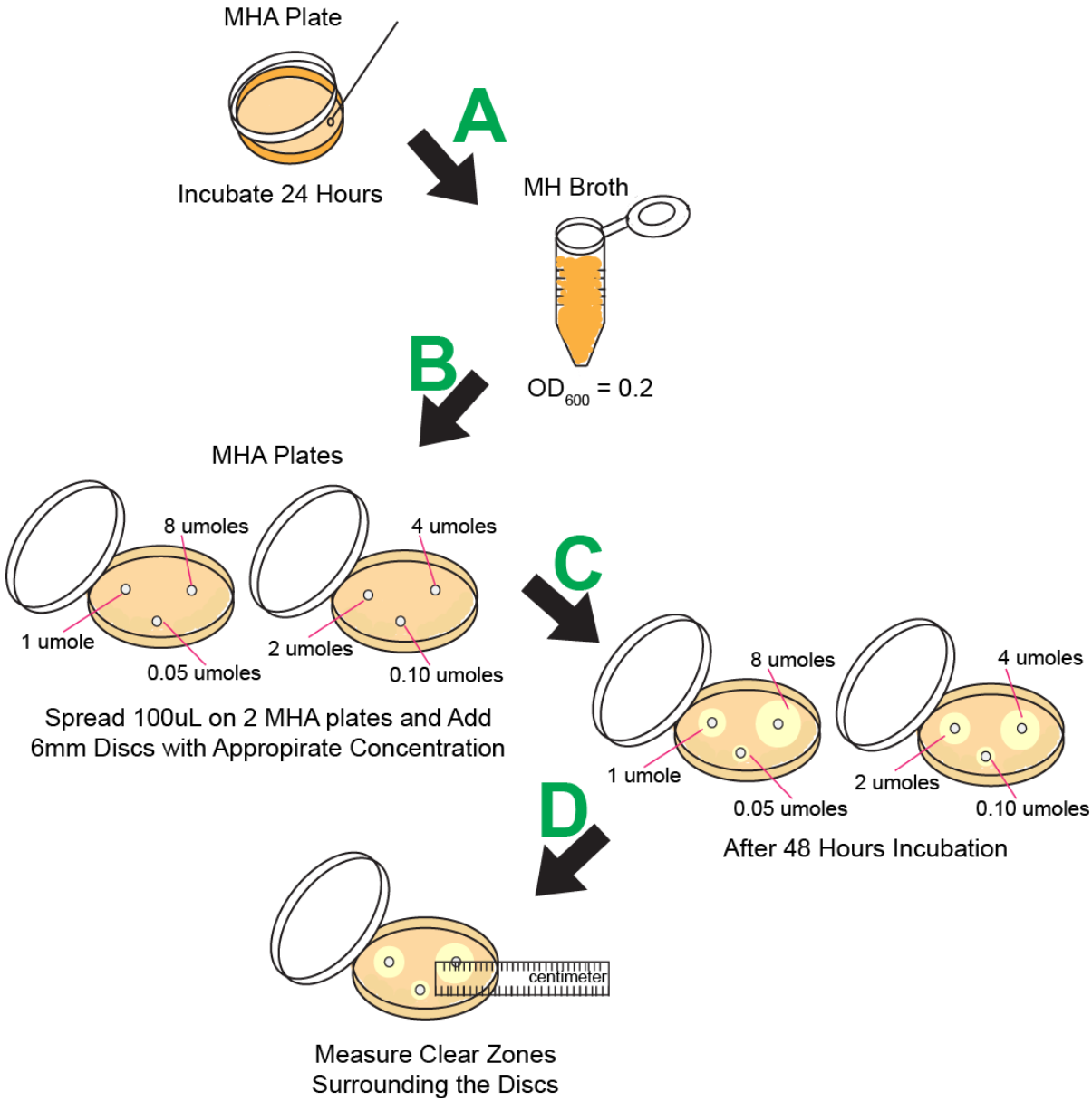

MHA Plate
A
Incubate 24 Hours
MH Broth
B
OD600 = 0.2
MHA Plates
8 umoles
4 umoles
1 umole
2 umoles
0.05 umoles
0.10 umoles
Spread 100uL on 2 MHA plates and Add
6mm Discs with Appropirate Concentration
C
8 umoles
4 umoles
1 umole
2 umoles
0.05 umoles
0.10 umoles
After 48 Hours Incubation
D
centimeter
Measure Clear Zones
Surrounding the Discs

Figure A3. Adhesion and invasion assay. Caco-2 cells were taken from frozen and put in flasks to grow. When the cells were confluent, they were split and separated into 24-well plates prior to incubating for 24 h (Step A1). Twenty-four hours cultures of *Campylobacter* strains were diluted to an OD_{600} of 0.2 (Step A2). Bacterial strains were placed over Caco-2 cells and plates were centrifuged prior to 2 h incubation (Steps B1 and B2). To measure the total amount of bacterial cells, samples were washed three times with EBSS and cells were lysed with Triton-X (Step C1). Suspensions were diluted in PBS (Step D1) and plated on CCDA plates (Step E1). For invasion cell count, Caco-2 cells were washed twice with EBSS before extra 2 h incubation with gentamicin. Cells were then washed with EBSS and lysed with Triton-X (Step C2) prior to diluting suspensions (Step D2) and plating them on CCDA plates (Step E2). The quantity of bacterial cells that adhered was determined by subtracting the total amount of cells by the number of invasive bacteria.

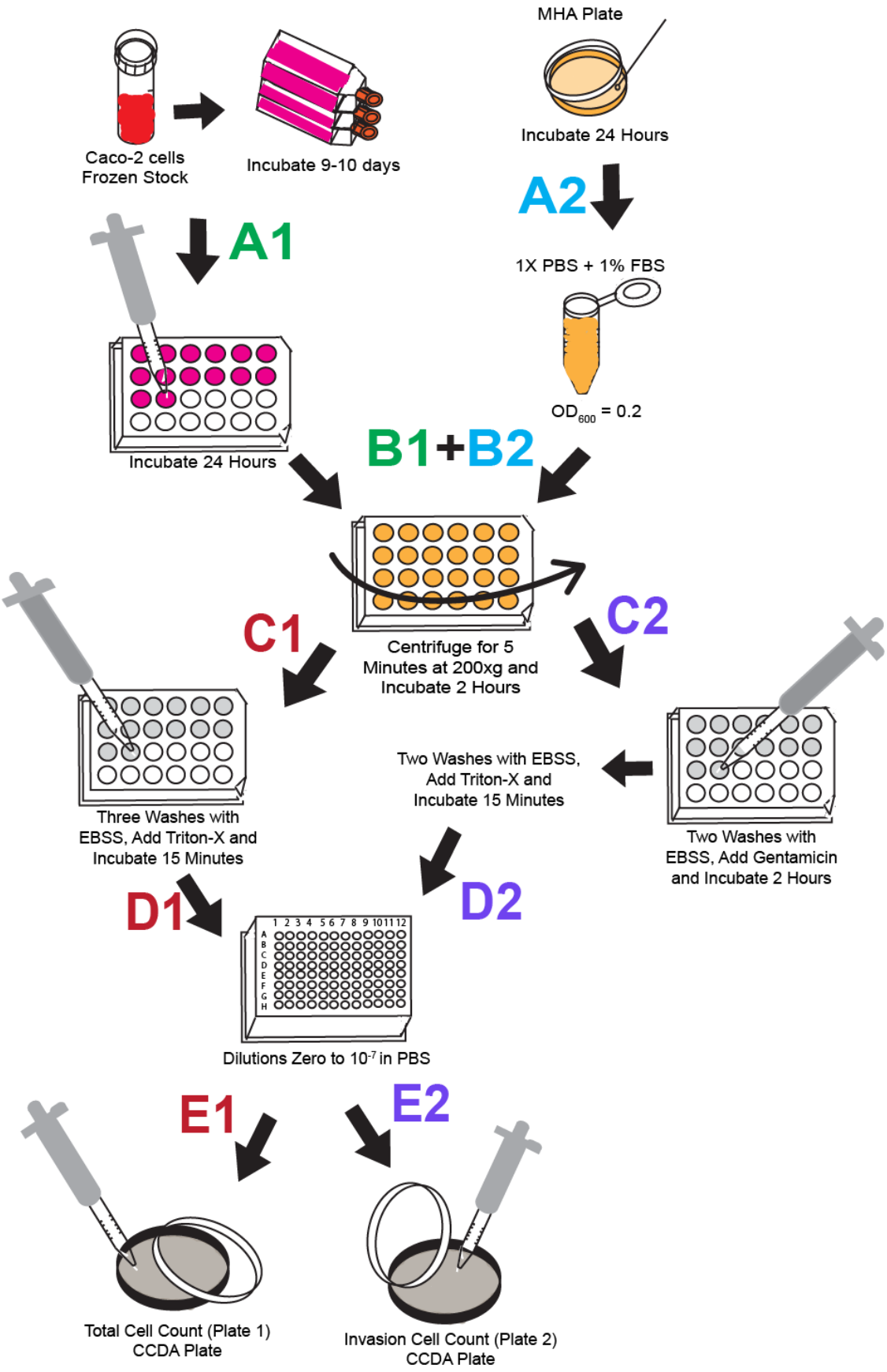

Caco-2 cells
Frozen Stock
Incubate 9-10 days
MHA Plate
Incubate 24 Hours
A2
A1
1X PBS + 1% FBS
OD600 = 0.2
Incubate 24 Hours
B1+B2
Centrifuge for 5
Minutes at 200xg and
Incubate 2 Hours
C1
C2
Three Washes with
EBSS, Add Triton-X and
Incubate 15 Minutes
Two Washes with EBSS,
Add Triton-X and
Incubate 15 Minutes
Two Washes with
EBSS, Add Gentamicin
and Incubate 2 Hours
D1
D2
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
Dilutions Zero to 10-7 in PBS
E1
E2
Total Cell Count (Plate 1)
CCDA Plate
Invasion Cell Count (Plate 2)
CCDA Plate

Figure A4. "RNA VS gDNA" microarray experiment. Total RNA and genomic DNA were extracted from *Campylobacter* strains. RNA was reverse transcribed and complementary DNA was labelled with Cy dyes while genomic DNA was first sonicated prior to labelling with a Cy dye. The labelled samples were combined and placed on a microarray slide to obtain the relative copy number for each gene.

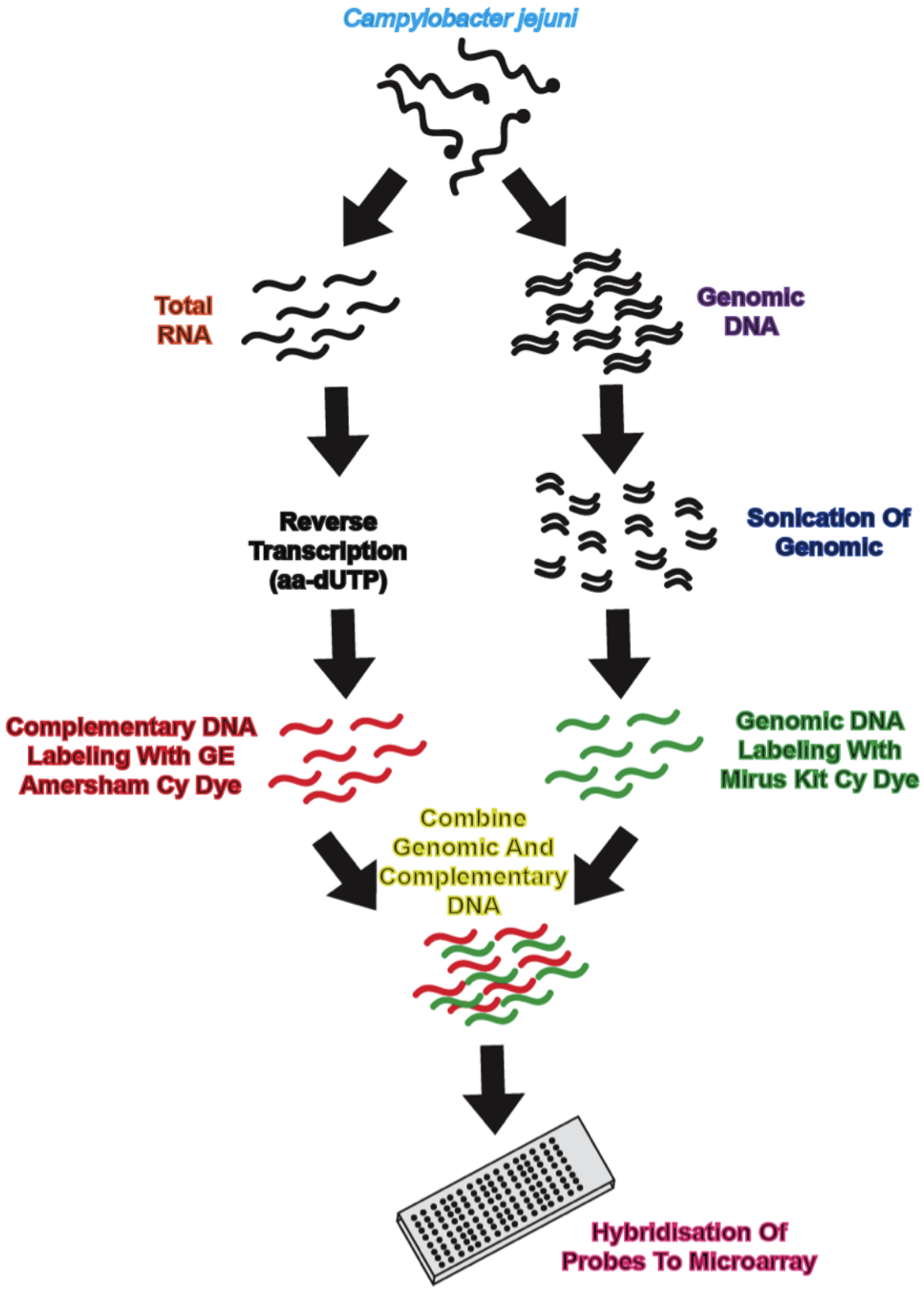

Campylobacter jejuni
Total RNA
Genomic DNA
Reverse Transcription (aa-dUTP)
Sonication Of Genomic
Complementary DNA Labeling With GE Amersham Cy Dye
Genomic DNA Labeling With Mirus Kit Cy Dye
Combine Genomic And Complementary DNA
Hybridisation Of Probes To Microarray

Figure A5. Sonication of genomic DNA protocol. Sonication buffer was added to DNA samples before sonicating (Step A). Sonicated DNA was first precipitated by incubating with glycogen, isopropanol and sodium acetate then samples were spun to recover pellets (Step B). After an ethanol wash (Step C), the pellet was resuspended in water and the concentration was measured using the Nanodrop (Step D). Sonicated DNA samples were run on a gel to visualize fragmentation (Step E).

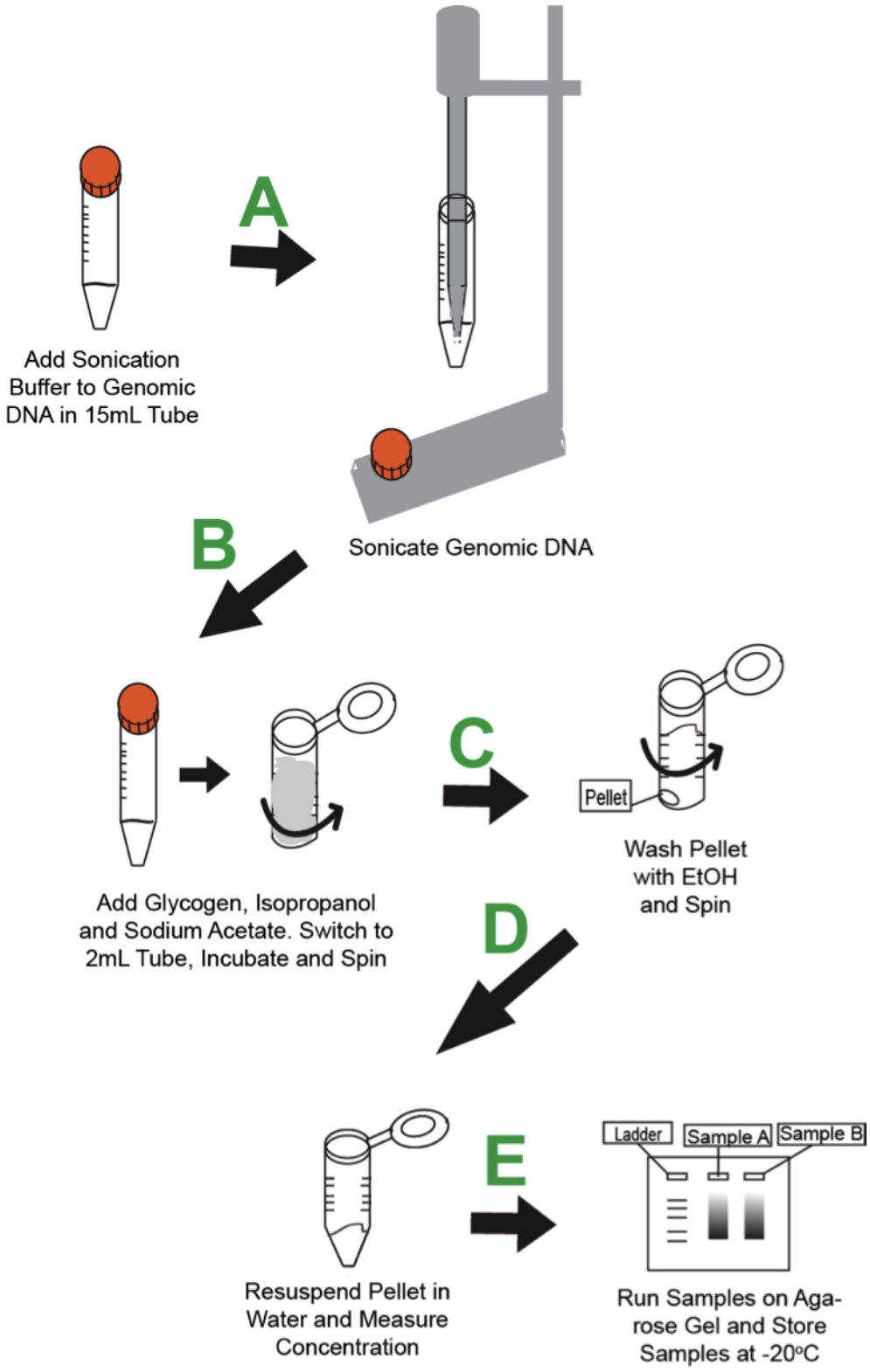

A
Add Sonication Buffer to Genomic DNA in 15mL Tube
Sonicate Genomic DNA
B
Add Glycogen, Isopropanol and Sodium Acetate. Switch to 2mL Tube, Incubate and Spin
C
Pellet
Wash Pellet with EtOH and Spin
D
E
Resuspend Pellet in Water and Measure Concentration
Ladder
Sample A
Sample B
Run Samples on Agarose Gel and Store Samples at -20°C

Table A2. Primers for real-time PCR experiments of *C. jejuni* phage-containing isolates. Primers were designed using Primer3plus program for real-time PCR using SybrGreen experiments. Genes were selected based on previous microarray results of *C. jejuni* phage-containing isolates and the control strain.

Primer	DNA sequence from 5’ to 3’
Cj0013_01	CGCTATGAAGGTCCAAAAGG
Cj0013_02	CGACCATCGGTGATTAAAGC
Cj0035c_01	TGTGGTTGGGGCTATTAACG
Cj0035c_02	CAACCAATACGCAAGCACAC
Cj0177_01	AAGCCATAAGCTAAATCCTGAAG
Cj0177_02	CCAATACATCAGCCATACGC
Cj0178_01	CGTGGTATGAGTGGTTTTGG
Cj0178_02	AGCTGAAGTTCCCGTTTGTG
Cj0311_01	GGGTGATGCTTTACTTGTTCG
Cj0311_02	CCTACAACCGCTACTCTATCAGC
Cj0332c_01	GATTAGCGGTCCTGTTGTTGTTTC
Cj0332c_02	AGCATTAGCGTCAATACTCTCAGC
Cj0333c_01	GCTTGTGGTTCTTGTATTGATGAG
Cj0333c_02	TGTCCTGATTCTACAGCACTCC
Cj0342c_01	CGTTCAAATCCTGCCACCTATAC
Cj0342c_02	CCGCTACATTTCTCACATCTTCC
Cj0345_01	GTGAGTGAAGTGTATGCCAATATG
Cj0345_02	ACCGCACCACCATAAATTCC
Cj0606_01	TGCTCCTATGGATGGTGTTG
Cj0606_02	TCTTACTCACATCAGCCTCTGC
Cj0951c_01	GGTCAATCAAGCAGGTAATATAC
Cj0951c_02	CACTGTTCTGAGCCAACTG
Cj1392_01	AACTTATTCATTGTGGCAGAGG
Cj1392_02	GCACGCATAAGTGTAGGATTG
Cj1638_01	GGATTAAAAGCTGCGACACG
Cj1638_02	AGCTCAGCAGGATCTTTTCC
Cj1663_01	ATCATGGGTCCATCAGGAAG
Cj1663_02	TTTTTCACGACGCAAGGTG

Table A3. Strains and plasmids for mutant construction. Plasmids were constructed for five *C. jejuni* genes *Cj0075c*, *Cj0199c*, *Cj0305c*, *Cj0809c* and *Cj1373*. When transforming these plasmids into *C. jejuni* NCTC11168, two plasmids, pGA0075c and pGA1373, were successfully transformed to produce the GA0075c and GA1373 *C. jejuni* mutant strains. Recombinant plasmids were transformed into *E. coli* DH5α. *Cm^r and cam* indicated the chloramphenicol resistance gene while *Amp^r* represents ampicillin resistance.

Strain	Description	Reference
E. coli DH5α	*endA1 hsdR17*(r_k^- m_k^-) *supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA argF U169 deoR* [ϕ*80dlac*Δ(*lacZ*Δ*M15*)]	Invitrogen
C. jejuni strains		
NCTC 11168	*C. jejuni* NCTC 11168	National Collection of Type Cultures
GA0075c	NCTC 11168ΔCj0075c	This study
GA1373	NCTC 11168ΔCj1373	This study
Plasmids		
pUC19	Cloning and suicide vector, Amp^r	Biolabs
pRY111	Cm^r resistance gene	(94)
pGA0075c	pUC19 carrying ΔCj0075c::cam	This study
pGA0199c	pUC19 carrying ΔCj0199c::cam	This study
pGA0305c	pUC19 carrying ΔCj0305c::cam	This study
pGA0809c	pUC19 carrying ΔCj0809c::cam	This study
pGA1373	pUC19 carrying ΔCj1373::cam	This study

Table A4. Primers for mutant construction. Primers for mutant construction were designed using the Primer3 program. The sequences complementary to pUC19 (Gene Cloning) and the CAT cassette (Inverse PCR) are in bold.

Primers	DNA sequence 5’ to 3’
For Gene Cloning	
Cj0075c-01	**CGGTACCCGGGGATCC**CAAGGAATCATCCCTGAAGTT
Cj0075c-02	**CGACTCTAGAGGATCC**CTTAAGCCCTGCCAGTCTTT
Cj0199c-01	**CGGTACCCGGGGATCC**CATTCTTCGGCAAACTACTTCA
Cj0199c-02	**CGACTCTAGAGGATCC**GCGAAAGGTCAAAGCTAGGC
Cj0305c-01	**CGGTACCCGGGGATCC**ATTCTACAGCCTTGCCACCT
Cj0305c-02	**CGACTCTAGAGGATCC**CTGCCACAACCAAATTCAAA
Cj0809c-01	**CGGTACCCGGGGATCC**CAGAATCAATCCCGCCACTA
Cj0809c-02	**CGACTCTAGAGGATCC**TCCTCCCACAGCTGAATAAA
Cj1373-01	**CGGTACCCGGGGATCC**CAAGGCTTTGATGCCCTACT
Cj1373-02	**CGACTCTAGAGGATCC**CAGCTTAGCGTGGATGAGAA
For Amplification of CAT cassette	
CAT-01	TGCTCGGCGGTGTTCCTTTCCAAG
CAT-02	TGCGCCCTTTAGTTCCTAAAGGGT
For Inverse PCR	
Cj0075c-03	**GAACTAAAGGGCGCA**AATGCTGTGGTTTTGGAGGA
Cj0075c-04	**GAACACCGCCGAGCA**TCATCATACCCGCACAAGAA
Cj0199c-03	**GAACTAAAGGGCGCA**CATACAAGGAAAATCCAATGAAA
Cj0199c-04	**GAACACCGCCGAGCA**TATCCGCCGTTTATGATGGT
Cj0305c-03	**GAACTAAAGGGCGCA**CGCTTTTTCTATGGGGGTTT
Cj0305c-04	**GAACACCGCCGAGCA**AAGCAAATCCCCCAAATACC
Cj0809c-03	**GAACTAAAGGGCGCA**GACACACACCAGGTTGCACT
Cj0809c-04	**GAACACCGCCGAGCA**AAAAATCCGCATCGCTAGAA
Cj1373-03	**GAACTAAAGGGCGCA**TTTTTGCTCTTATGGGGCTTT
Cj1373-04	**GAACACCGCCGAGCA**TTCATTTTGGGCTTTTGTGC

Appendix B: Tables for results and discussion

Table B1. Cluster information for strains from clinical, animal and water sources. Clinical, animal and water strains used in this study were chosen from a larger pool of isolates separated into clusters by Comparative Genomic Fingerprinting (CGF) genotype. When considering the larger pool of strains, some clusters were associated with various sources, whereas others were limited to isolates from only one source. When comparing phenotypic results in this study, the analysis was based on clusters (animal, water, clinical and animal) and sources (clinical, animal, water) with respect to the 67 strains analysed only. For instance, cluster 179 consisted of strains from clinical, animal and water isolates in the larger pool of strains and strains from cluster 179 chosen for this analysis were representative of all sources.

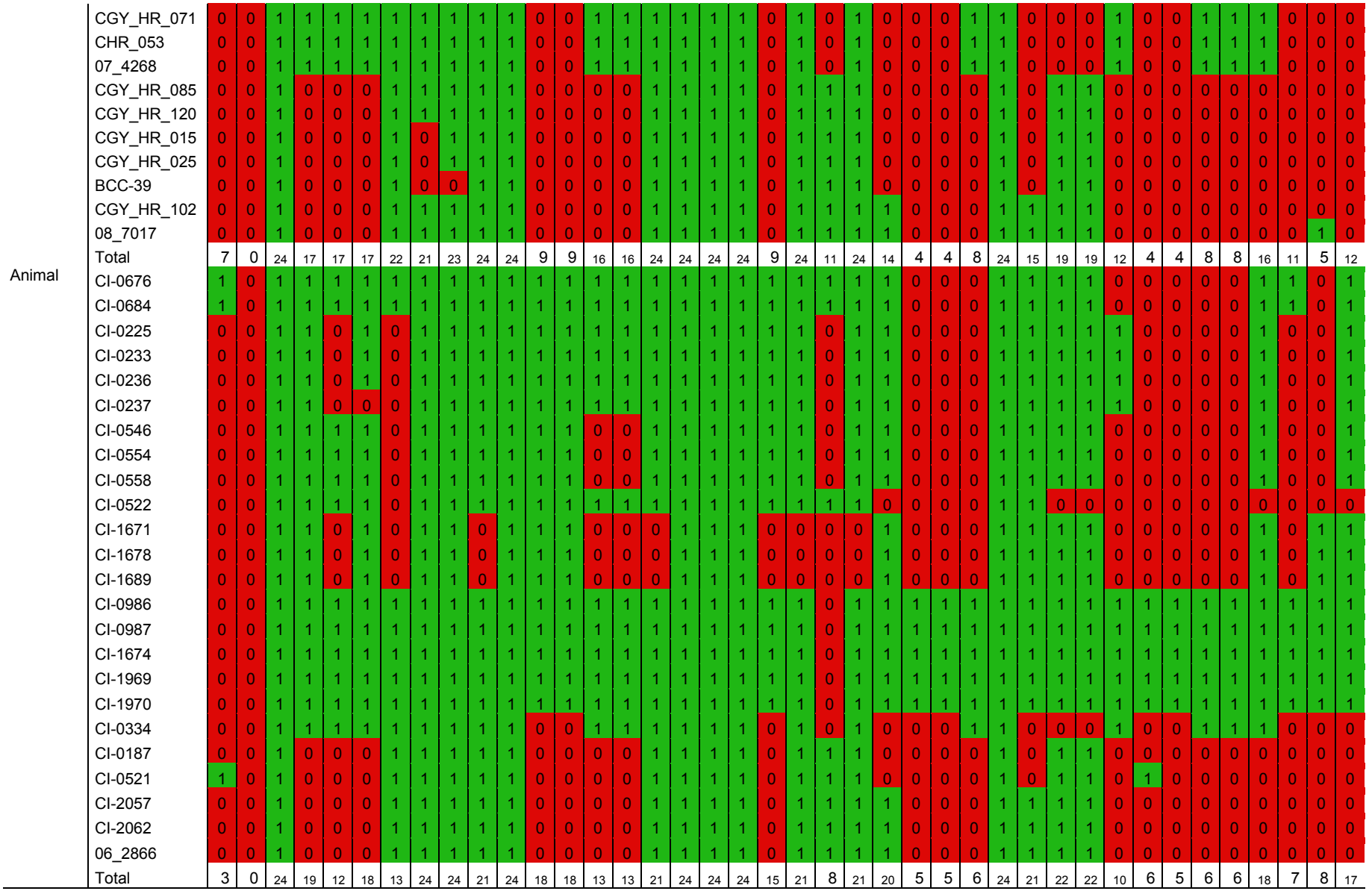
Cluster	Associations of the larger pool of strains	Used in Cluster	Used in Source
10	All C. coli strains from chicken and pig.	Animal	Animal
103	All water strains from Oldman River Basin.	Water	Water
179	Primarily clinical with some cow and water isolates.	N/A	Animal, Clinical, Water
231	Primarily water with some avian isolates.	Water	Water
242	Primarily water with some clinical, duck and geese isolates.	Water	Water
332	Primarily clinical with some water, chicken and sheep isolates.	N/A	Animal, Clinical, Water
337	Primarily clinical with some chicken, goose, duck, dogs and water isolates.	N/A	Animal, Clinical, Water
396	Primarily clinical strains and a water isolate strongly linked with clinical isolates.	N/A	Clinical, Water
409	Primarily clinical strains with some chicken isolates.	Clinical and Animal	Animal, Clinical
423	All chicken isolates.	Animal	Animal
438	Primarily chicken with some clinical isolates.	Animal	Animal, Clinical
516	1/3 clinical with some water, chicken and cow isolates.	N/A	Animal, Clinical, Water
540	Clinical, water and cow isolates.	N/A	Water
563	Primarily clinical with some chicken and cow isolates.	Clinical and Animal	Animal, Clinical

Table B2. Comparative Genomic Fingerprinting results for strains sorted by clusters.

Clusters	Strain	cj0008	cj0033	cj0035	cj0057	cj0177	cj0181	cj0264c	cj0297c	cj0298c	cj0307	cj0421c	cj0483	cj0486	cj0566	cj0569	cj0570	cj0625	cj0728	cj0733	cj0755	cj0763	cj0860	cj0967	cj1134	cj1136	cj1141	cj1151c	cj1294	cj1324	cj1329	cj1334	cj1427c	cj1431c	cj1439c	cj1550c	cj1552	cj1585	cj1679	cj1721	cj1727c
Water	CI-0109	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	CI-0310	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	CI-0475	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	CI-0532	0	0	1	1	0	0	0	0	0	1	1	0	0	1	1	1	1	1	1	0	1	0	1	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
	CI-3039	0	0	1	1	0	0	0	0	0	1	1	0	0	1	1	1	1	1	1	0	1	0	1	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
	CI-3000	0	1	1	1	0	0	0	0	0	1	1	0	0	1	1	1	1	1	1	0	1	0	1	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
	CI-0599	0	1	1	1	0	0	0	0	0	1	1	0	0	0	0	1	1	1	0	0	0	0	1	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0
	CI-1446	0	1	1	1	0	0	0	0	0	1	1	0	0	0	0	1	1	1	0	0	0	0	1	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0
	CI-1814	0	1	1	1	0	0	0	0	0	1	1	0	0	0	0	1	1	1	0	0	0	0	1	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0
	CI-2995	0	1	1	1	0	0	0	0	0	1	1	0	0	0	0	1	1	1	0	0	0	0	1	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0
	CI-0571	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0	0
	CI-0578	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0	0
	Total	2	7	10	9	2	2	2	2	2	9	9	5	2	5	5	9	9	9	5	4	5	0	9	6	2	2	2	9	7	9	9	0	0	0	2	2	2	2	0	0
Animal	CI-0225	0	0	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	0	1	1	1	1	1	0	0	0	0	1	0	0	1
	CI-0233	0	0	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	0	1	1	1	1	1	0	0	0	0	1	0	0	1
	CI-0236	0	0	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	0	1	1	1	1	1	0	0	0	0	1	0	0	1
	CI-0237	0	0	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	0	1	1	1	1	1	0	0	0	0	1	0	0	1
	CGY_HR_072	0	0	1	1	1	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	1	0	0	0	1	1	1	1	0	0	0	0	0	1	0	0	1
	CI-0546	0	0	1	1	1	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	1	0	0	0	1	1	1	1	0	0	0	0	0	1	0	0	1
	CI-0554	0	0	1	1	1	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	1	0	0	0	1	1	1	1	0	0	0	0	0	1	0	0	1
	CI-0558	0	0	1	1	1	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	1	0	0	0	1	1	1	1	0	0	0	0	0	1	0	0	1
	CI-1671	0	0	1	1	0	1	0	1	1	0	1	1	1	0	0	0	1	1	1	0	0	0	0	1	0	0	0	1	1	1	1	0	0	0	0	0	1	0	1	1
	CI-1678	0	0	1	1	0	1	0	1	1	0	1	1	1	0	0	0	1	1	1	0	0	0	0	1	0	0	0	1	1	1	1	0	0	0	0	0	1	0	1	1
	CI-1689	0	0	1	1	0	1	0	1	1	0	1	1	1	0	0	0	1	1	1	0	0	0	0	1	0	0	0	1	1	1	1	0	0	0	0	0	1	0	1	1
	Total	0	0	11	11	4	10	0	11	11	8	11	11	11	4	4	8	11	11	11	8	8	0	8	11	0	0	0	11	11	11	11	4	0	0	0	0	11	0	3	11
Clinical and Animal	BCC-67	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0	0	0	0	0	1	1	0	1
	CGY_HR_082	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0	0	0	0	0	1	1	0	1
	CGY_HR_090	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0	0	0	0	0	1	1	0	1
	CGY_HR_098	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0	0	0	0	0	1	1	0	1
	CI-0676	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0	0	0	0	0	1	1	0	1
	CI-0684	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0	0	0	0	0	1	1	0	1
	CGY_HR_109	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	CGY_HR_130	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	CHR_037	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	CHR_151	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	CI-0986	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	CI-0987	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	CI-1674	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	CI-1969	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	CI-1970	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	Total	6	0	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	6	15	15	9	9	9	15	15	15	15	9	9	9	9	9	15	15	9	15

Table B3. Comparative Genomic Fingerprinting results sorted by sources.

Sources	Strain	cj0008	cj0033	cj0035	cj0057	cj0177	cj0181	cj0264c	cj0297c	cj0298c	cj0307	cj0421c	cj0483	cj0486	cj0566	cj0569	cj0570	cj0625	cj0728	cj0733	cj0755	cj0763	cj0860	cj0967	cj1134	cj1136	cj1141	cj1151c	cj1294	cj1324	cj1329	cj1334	cj1427c	cj1431c	cj1439c	cj1550c	cj1552	cj1585	cj1679	cj1721	cj1727c
Water	CI-2200	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	0	1	1	0	0	0	1	1	1	1	1	0	0	0	0	1	1	0	1
	CI-1415	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
	CI-2822	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	CI-0571	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0	0
	CI-0578	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0	0
	CI-0292	0	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	0	1	0	0	0	1	1	0	0	0	1	0	0	1	1	1	0	0	0
	CI-0616	0	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	0	1	0	0	0	1	1	0	0	0	1	0	0	1	1	1	0	0	0
	CI-2916	0	0	1	0	0	0	1	1	1	1	1	0	0	0	0	1	1	1	1	0	1	1	1	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
	CI-2847	0	0	1	0	0	0	1	1	1	1	1	0	0	0	0	1	1	1	1	0	1	1	1	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0
	CI-0532	0	0	1	1	0	0	0	0	0	1	1	0	0	1	1	1	1	1	1	0	1	0	1	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
	CI-3039	0	0	1	1	0	0	0	0	0	1	1	0	0	1	1	1	1	1	1	0	1	0	1	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
	CI-3000	0	1	1	1	0	0	0	0	0	1	1	0	0	1	1	1	1	1	1	0	1	0	1	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
	CI-0599	0	1	1	1	0	0	0	0	0	1	1	0	0	0	0	1	1	1	0	0	0	0	1	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0
	CI-1446	0	1	1	1	0	0	0	0	0	1	1	0	0	0	0	1	1	1	0	0	0	0	1	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0
	CI-1814	0	1	1	1	0	0	0	0	0	1	1	0	0	0	0	1	1	1	0	0	0	0	1	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0
	CI-2995	0	1	1	1	0	0	0	0	0	1	1	0	0	0	0	1	1	1	0	0	0	0	1	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0
	CI-0109	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	CI-0310	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	CI-0475	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	Total	3	7	17	14	7	7	8	9	9	16	16	7	4	10	10	16	16	16	12	6	12	3	16	9	2	2	5	16	11	13	13	4	1	1	5	5	6	4	1	2
Clinical	BCC-67	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0	0	0	0	0	1	1	0	1
	CGY_HR_082	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0	0	0	0	0	1	1	0	1
	CGY_HR_098	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0	0	0	0	0	1	1	0	1
	BCC-62	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	0	1	1	0	0	0	1	1	1	1	1	0	0	0	0	1	1	0	1
	BCC-70	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	0	1	1	0	0	0	1	1	1	1	1	0	0	0	0	1	1	0	1
	CGY_HR_108	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	0	1	1	0	0	0	1	1	1	1	1	0	0	0	0	1	1	0	1
	CGY_HR_208	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	0	1	1	0	0	0	1	1	1	1	1	0	0	0	0	1	1	0	1
	CGY_HR_072	0	0	1	1	1	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	1	0	0	0	1	1	1	1	0	0	0	0	0	1	0	0	1
	CGY_HR_074	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
	CGY_HR_109	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	CGY_HR_130	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	CHR_037	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	CHR_151	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	CGY_HR_058	0	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	0	1	0	0	0	1	1	0	0	0	1	0	0	1	1	1	0	0	0


Animal
CGY_HR_071 0 0 1 1 1 1 1 1 1 1 1 0 0 1 1 1 1 1 1 0 1 0 1 0 0 0 1 1 0 0 0 1 0 0 1 1 1 0 0 0
CHR_053 0 0 1 1 1 1 1 1 1 1 1 0 0 1 1 1 1 1 1 0 1 0 1 0 0 0 1 1 0 0 0 1 0 0 1 1 1 0 0 0
07_4268 0 0 1 1 1 1 1 1 1 1 1 0 0 1 1 1 1 1 1 0 1 0 1 0 0 0 1 1 0 0 0 1 0 0 1 1 1 0 0 0
CGY_HR_085 0 0 1 0 0 0 1 1 1 1 1 0 0 0 0 1 1 1 1 0 1 1 1 0 0 0 0 1 0 1 1 0 0 0 0 0 0 0 0 0
CGY_HR_120 0 0 1 0 0 0 1 1 1 1 1 0 0 0 0 1 1 1 1 0 1 1 1 0 0 0 0 1 0 1 1 0 0 0 0 0 0 0 0 0
CGY_HR_015 0 0 1 0 0 0 1 0 1 1 1 0 0 0 0 1 1 1 1 0 1 1 1 0 0 0 0 1 0 1 1 0 0 0 0 0 0 0 0 0
CGY_HR_025 0 0 1 0 0 0 1 0 1 1 1 0 0 0 0 1 1 1 1 0 1 1 1 0 0 0 0 1 0 1 1 0 0 0 0 0 0 0 0 0
BCC-39 0 0 1 0 0 0 1 0 0 1 1 0 0 0 0 1 1 1 1 0 1 1 1 0 0 0 0 1 0 1 1 0 0 0 0 0 0 0 0 0
CGY_HR_102 0 0 1 0 0 0 1 1 1 1 1 0 0 0 0 1 1 1 1 0 1 1 1 1 0 0 0 1 1 1 1 0 0 0 0 0 0 0 0 0
08_7017 0 0 1 0 0 0 1 1 1 1 1 0 0 0 0 1 1 1 1 0 1 1 1 1 0 0 0 1 1 1 1 0 0 0 0 0 0 0 1 0
Total 7 0 24 17 17 17 22 21 23 24 24 9 9 16 16 24 24 24 24 9 24 11 24 14 4 4 8 24 15 19 19 12 4 4 8 8 16 11 5 12
CI-0676 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 0 1 1 1 1 0 0 0 0 0 1 1 0 1
CI-0684 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 0 1 1 1 1 0 0 0 0 0 1 1 0 1
CI-0225 0 0 1 1 0 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 0 0 0 1 1 1 1 1 0 0 0 0 1 0 0 1
CI-0233 0 0 1 1 0 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 0 0 0 1 1 1 1 1 0 0 0 0 1 0 0 1
CI-0236 0 0 1 1 0 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 0 0 0 1 1 1 1 1 0 0 0 0 1 0 0 1
CI-0237 0 0 1 1 0 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 0 0 0 1 1 1 1 1 0 0 0 0 1 0 0 1
CI-0546 0 0 1 1 1 1 0 1 1 1 1 1 1 0 0 1 1 1 1 1 1 0 1 1 0 0 0 1 1 1 1 0 0 0 0 0 1 0 0 1
CI-0554 0 0 1 1 1 1 0 1 1 1 1 1 1 0 0 1 1 1 1 1 1 0 1 1 0 0 0 1 1 1 1 0 0 0 0 0 1 0 0 1
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CI-0522 0 0 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0
CI-1671 0 0 1 1 0 1 0 1 1 0 1 1 1 0 0 0 1 1 1 0 0 0 0 1 0 0 0 1 1 1 1 0 0 0 0 0 1 0 1 1
CI-1678 0 0 1 1 0 1 0 1 1 0 1 1 1 0 0 0 1 1 1 0 0 0 0 1 0 0 0 1 1 1 1 0 0 0 0 0 1 0 1 1
CI-1689 0 0 1 1 0 1 0 1 1 0 1 1 1 0 0 0 1 1 1 0 0 0 0 1 0 0 0 1 1 1 1 0 0 0 0 0 1 0 1 1
CI-0986 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
CI-0987 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
CI-1674 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
CI-1969 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
CI-1970 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
CI-0334 0 0 1 1 1 1 1 1 1 1 1 0 0 1 1 1 1 1 1 0 1 0 1 0 0 0 1 1 0 0 0 1 0 0 1 1 1 0 0 0
CI-0187 0 0 1 0 0 0 1 1 1 1 1 0 0 0 0 1 1 1 1 0 1 1 1 0 0 0 0 1 0 1 1 0 0 0 0 0 0 0 0 0
CI-0521 1 0 1 0 0 0 1 1 1 1 1 0 0 0 0 1 1 1 1 0 1 1 1 0 0 0 0 1 0 1 1 0 1 0 0 0 0 0 0 0
CI-2057 0 0 1 0 0 0 1 1 1 1 1 0 0 0 0 1 1 1 1 0 1 1 1 1 0 0 0 1 1 1 1 0 0 0 0 0 0 0 0 0
CI-2062 0 0 1 0 0 0 1 1 1 1 1 0 0 0 0 1 1 1 1 0 1 1 1 1 0 0 0 1 1 1 1 0 0 0 0 0 0 0 0 0
06_2866 0 0 1 0 0 0 1 1 1 1 1 0 0 0 0 1 1 1 1 0 1 1 1 1 0 0 0 1 1 1 1 0 0 0 0 0 0 0 0 0
Total 3 0 24 19 12 18 13 24 24 21 24 18 18 13 13 21 24 24 24 15 21 8 21 20 5 5 6 24 21 22 22 10 6 5 6 6 18 7 8 17

Table B4. The top 10 most adherent and invasive *Campylobacter* clinical, animal and water strains.

Adhesion					Invasion				
Source	Strain	Cell count	Cluster	Related to clinical?	Source	Strain	Cell count	Cluster	Related to clinical?
Animal	06-2866	3.95E+07	337	Yes	Animal	06-2866	6.92E+04	337	Yes
	CI-2057	6.78E+06	337	Yes		CI-0684	5.13E+03	409	Yes
	CI-0237	4.90E+06	423	No		CI-0334	4.00E+03	179	Yes
	CI-0676	4.82E+06	409	Yes		CI-0676	4.00E+03	409	Yes
	CI-1970	3.80E+06	563	Yes		CI-2057	3.15E+03	337	Yes
	CI-1689	3.02E+06	10	No		CI-1689	1.60E+03	10	No
	CI-1969	2.58E+06	563	Yes		CI-0236	1.51E+03	423	No
	CI-1678	2.56E+06	10	No		CI-0987	1.11E+03	563	Yes
	CI-0233	2.10E+06	423	No		CI-1970	1.00E+03	563	Yes
	CI-0986	2.05E+06	563	Yes		CI-2062	7.33E+02	337	Yes
Water	CI-2822	1.87E+07	563	Yes	Water	CI-0109	5.59E+04	103	No
	CI-0475	1.55E+07	103	No		CI-0475	3.67E+04	103	No
	CI-0571	1.49E+07	540	Yes		CI-2822	1.29E+04	563	Yes
	CI-2995	1.05E+07	231	No		CI-2995	7.46E+03	231	No
	CI-2200	6.82E+06	396	Yes		CI-0616	6.02E+03	179	Yes
	CI-0292	4.89E+06	179	Yes		CI-0292	4.30E+03	179	Yes
	CI-1446	2.42E+06	231	No		CI-1446	2.83E+03	231	No
	CI-0578	2.19E+06	540	Yes		CI-0571	2.53E+03	540	Yes
	CI-0616	1.88E+06	179	Yes		CI-1415	2.11E+03	516	Yes
	CI-0109	1.62E+06	103	No		CI-2847	1.11E+03	337	Yes
Clinical	CGY_HR_072	1.07E+07	438	N/A	Clinical	CGY_HR_071	6.56E+04	179	N/A
	CGY_HR_058	1.01E+07	179	N/A		CGY_HR_098	1.82E+04	409	N/A
	CGY_HR_098	7.12E+06	409	N/A		BCC-67	1.49E+04	409	N/A
	BCC-70	6.42E+06	396	N/A		CGY_HR_102	1.27E+04	337	N/A
	CGY_HR_102	6.39E+06	337	N/A		CHR_053	9.11E+03	179	N/A
	CHR_151	4.54E+06	563	N/A		CGY_HR_082	6.00E+03	409	N/A
	BCC-67	4.32E+06	409	N/A		CGY_HR_025	4.77E+03	332	N/A
	CHR_053	4.01E+06	179	N/A		CGY_HR_085	3.78E+03	332	N/A
	BCC-62	3.74E+06	396	N/A		08-7017	3.33E+03	337	N/A
	07-4268	3.67E+06	179	N/A		BCC-62	3.00E+03	396	N/A

Appendix C: Microarray data

Table C1. Genes present with statistically significant differences between water and clinical strains. Seventy-four genes had significant differences (in presence) between clinical and water strains. Genes *Cj0011c*, *Cj0033*, *Cj1248*, *Cj1642* and 3 RM1221 proteins (CJE0273, CJE0732, CJE1550) were more prevalent in water strains while the rest of the genes had a higher number of clinical expressing them. Percentage of strains was obtained by dividing the number of strains in a source carrying the gene with the total number of strains in the source.

Gene	% Present in water	% Present in clinical	Annotation
atpH	67	100	ATP synthase subunit delta
CJE1284	33	0	Putative lipooligosaccharide biosynthesis glycosyltransferase
CJE1550	0	38	Hypothetical protein
CJE1732	0	46	Arsenate reductase
CJE0273	0	54	Hypothetical protein
CJE0550	58	0	Putative site-specific DNA-methyltransferase
Cj0007	58	100	Glutamate synthase (NADPH) large subunit
Cj0011c	75	31	Putative non-specific DNA binding protein
Cj0021c	33	77	Putative fumarylacetoacetate (FAA) hydrolase family protein
Cj0022c	67	100	Putative ribosomal pseudouridine synthase
Cj0033	58	15	Putative integral membrane protein
Cj0044c	17	62	Hypothetical protein
Cj0045c	58	100	Putative iron-binding protein
Cj0079c	67	100	Cytolethal-distending toxin A
Cj0120	58	100	Putative recombination protein RecO
Cj0126c	50	100	Conserved hypothetical protein Cj0126c
Cj0151c	58	100	Putative periplasmic protein
Cj0186c	67	100	Putative TerC family integral membrane protein
Cj0187c	50	92	Phosphoribosylglycinamide formyltransferase
Cj0190c	67	100	Conserved hypothetical protein Cj0190c
Cj0201c	50	92	Putative integral membrane protein
Cj0261c	50	100	Putative SAM-dependent methyltransferase
Cj0263	67	100	Zinc transporter ZupT
Cj0324	25	77	Ubiquinone/menaquinone biosynthesis methyltransferase
Cj0336c	42	85	Flagellar motor protein MotB
Cj0347	42	85	N-(5'-phosphoribosyl)anthranilate isomerase
Cj0379c	33	85	Putative sulfite oxidase subunit YedY
Cj0396c	50	92	Putative lipoprotein
Cj0525c	33	85	Putative penicillin-binding protein
Cj0589	58	100	Bifunctional riboflavin kinase/FMN adenylyltransferase
Cj0622	50	100	Carbamoyltransferase
Cj0637c	50	100	Methionine sulfoxide reductase A
Cj0661c	58	100	GTP-binding protein Era
Cj0730	58	100	Putative ABC transport system permease
Cj0731	67	100	Putative ABC transport system permease
Cj0736	67	100	Hypothetical protein
Cj0778	50	92	Major antigenic peptide PEB2

Cj0799c	58	100	Putative Holliday junction ATP-dependent DNA helicase
Cj0813	58	100	3-deoxy-manno-octulosonate cytidylyltransferase
Cj0822	67	100	Bifunctional phosphopantothenoylcysteine decarboxylase/phosphopantothenate synthase
Cj0851c	67	100	Putative integral membrane protein
Cj0890c	67	100	Putative sensory transduction transcriptional regulator
Cj0902	50	92	Putative glutamine transport ATP-binding protein
Cj0923c	25	85	Putative MCP protein methyltransferase
Cj0940c	25	69	Putative glutamine transport system permease
Cj0947c	67	100	Putative carbon-nitrogen hydrolase
Cj0951c	50	100	Putative MCP-domain signal transduction protein
Cj1013c	42	92	Putative cytochrome C biogenesis protein
Cj1023c	67	100	Aspartate-semialdehyde dehydrogenase
Cj1102	42	92	tRNA pseudouridine synthase B
Cj1163c	67	100	Putative cation transport protein
Cj1168c	58	100	Putative integral membrane protein (dedA homolog)
Cj1172c	67	100	Hypothetical protein
Cj1218c	67	100	Riboflavin synthase subunit alpha
Cj1222c	67	100	Two-component sensor (histidine kinase)
Cj1244	33	77	Putative radical SAM domain protein
Cj1248	42	0	GMP synthase
Cj1285c	67	100	Conserved hypothetical protein Cj1285c
Cj1348c	67	100	Putative coiled-coil protein
Cj1356c	58	100	Putative integral membrane protein
Cj1408	50	92	Flagellar basal body-associated protein FliL
Cj1418c	58	100	Hypothetical protein
Cj1443c	50	92	D-arabinose 5-phosphate isomerase
Cj1509c	67	100	Putative formate dehydrogenase, cytochrome B subunit
Cj1521c	42	85	Putative CRISPR-associated protein
Cj1642	75	31	Hypothetical protein
CJE0757	67	100	Di-/tripeptide transporter
CJE1611	8	54	GDP-mannose 4,6-dehydratase
CJE1733	0	46	Arsenical-resistance protein, putative
neuA2	58	100	Acylneuraminate cytidylyltransferase
pTet_01	0	46	Hypothetical protein
pTet_04	0	46	Hypothetical protein
pTet_16	0	38	Similar to ATPase
pVir_Cjp44	67	100	Unknown

Table C2. Genes present with statistically significant differences between strains from animal and water sources. There were 191 genes with significant differences (in presence) between animal and water strains. All the genes were more prevalent in animal strains. Percentage of strains was obtained by dividing the number of strains in a source carrying the gene with the total number of strains in the source.

Gene	% Present in animal	% Present in water	Annotation
acpS	71	8	4-phosphopantetheinyl transferase
atpF1	100	67	ATP synthase subunit B
atpH	100	67	ATP synthase subunit delta
CJE1420	36	0	Hypothetical protein
CJE1421	36	0	Site-specific DNA-methyltransferase
CJE1423	36	0	Hypothetical protein
CJE1426	36	0	Hypothetical protein
CJE1430	36	0	Putative RloG protein
CJE1435	36	0	DNA-binding protein Roi
CJE1444	36	0	Hypothetical protein
CJE1453	36	0	Hypothetical protein
CJE1455	36	0	Hypothetical protein
CJE1459	36	0	Hypothetical protein
CJE1460	43	0	Hypothetical protein
CJE1466	36	0	Hypothetical protein
CJE1468	36	0	HK97 family phage protein
CJE1471	36	0	Putative phage terminase small subunit
CJE1550	36	0	Hypothetical protein
CJE1732	50	0	Arsenate reductase
CJE0217	36	0	Hypothetical protein
CJE0234	50	8	Putative baseplate assembly protein W
CJE0241	50	8	Hypothetical protein
CJE0254	50	8	Putative tail protein D
CJE0273	36	0	Hypothetical protein
Cj0022c	100	67	Putative ribosomal pseudouridine synthase
Cj0038c	93	50	Putative poly(A) polymerase family protein
Cj0044c	86	17	Hypothetical protein
Cj0045c	100	58	Putative iron-binding protein
Cj0054c	93	50	Putative lysine decarboxylase family protein
Cj0079c	100	67	Cytolethal-distending toxin A
Cj0119	79	17	Putative hydrolase
Cj0126c	100	50	Conserved hypothetical protein Cj0126c
Cj0128c	79	33	Putative inositol monophosphatase family protein
Cj0137	79	25	Ribosome-binding factor A
Cj0151c	100	58	Putative periplasmic protein
Cj0157c	86	42	Putative integral membrane protein
Cj0160c	86	42	Putative radical SAM domain protein
Cj0186c	100	67	Putative TerC family integral membrane protein
Cj0187c	93	50	Phosphoribosylglycinamide formyltransferase
Cj0201c	93	50	Putative integral membrane protein

Cj0244	93	42	50S ribosomal protein L35
Cj0245	100	58	50S ribosomal protein L20
Cj0246c	93	33	Putative MCP-domain signal transduction protein
Cj0261c	93	50	Putative SAM-dependent methyltransferase
Cj0270	43	0	Putative tautomerase family protein
Cj0271	93	42	Bacterioferritin comigratory protein homolog
Cj0286c	93	50	Hypothetical protein
Cj0295	71	25	Putative acetyltransferase
Cj0302c	79	25	Putative molybdenum-pterin binding protein
Cj0310c	57	8	Putative efflux protein
Cj0324	71	25	Ubiquinone/menaquinone biosynthesis methyltransferase
Cj0327	50	8	Putative endoribonuclease L-PSP family protein
Cj0336c	86	42	Flagellar motor protein MotB
Cj0347	93	42	N-(5'-phosphoribosyl)anthranilate isomerase
Cj0350	64	17	Hypothetical protein
Cj0375	50	8	Putative lipoprotein
Cj0382c	86	25	Transcription antitermination protein NusB
Cj0396c	93	50	Putative lipoprotein
Cj0400	71	17	Ferric uptake regulator
Cj0436	93	50	Putative pyridoxamine 5'-phosphate oxidase
Cj0465c	93	50	Group III truncated haemoglobin
Cj0480c	93	33	Putative transcriptional regulator
Cj0481	93	42	Putative dihydrodipicolinate synthase
Cj0483	93	42	Putative altronate hydrolase C-terminus
Cj0484	100	42	Putative MFS (Major Facilitator Superfamily) transport protein
Cj0485	86	17	Short chain dehydrogenase
Cj0490	86	33	Putative aldehyde dehydrogenase C-terminus
Cj0523	86	33	Hypothetical protein
Cj0525c	100	33	Putative penicillin-binding protein
Cj0540	86	25	Putative exporting protein
Cj0568	43	0	Hypothetical protein
Cj0589	100	58	Bifunctional riboflavin kinase/FMN adenylyltransferase
Cj0622	93	50	Carbamoyltransferase
Cj0624	100	67	Hydrogenase isoenzymes formation protein
Cj0637c	100	50	Methionine sulfoxide reductase A
Cj0647	86	42	Putative HAD-superfamily hydrolase
Cj0690c	86	42	Putative restriction/modification enzyme
Cj0691	86	42	Hypothetical protein
Cj0703	71	17	Hypothetical protein
Cj0706	100	58	Conserved hypothetical protein Cj0706
Cj0710	86	8	30S ribosomal protein S16

Cj0712	86	42	Putative 16S rRNA processing protein
Cj0721c	93	50	Putative integral membrane protein
Cj0729	93	42	Putative type I phosphodiesterase/nucleotide pyrophosphatase
Cj0731	100	67	Putative ABC transport system permease
Cj0741	86	33	Hypothetical protein
Cj0766c	100	50	Thymidylate kinase
Cj0778	100	50	Major antigenic peptide PEB2
Cj0785	100	42	NapD protein homolog
Cj0799c	100	58	Putative Holliday junction ATP-dependent DNA helicase
Cj0800c	86	25	Putative ATPase
Cj0811	86	33	Tetraacyldisaccharide 4'-kinase
Cj0813	100	58	3-deoxy-manno-octulosonate cytidylyltransferase
Cj0815	50	8	Hypothetical protein
Cj0822	100	67	Bifunctional phosphopantothenoylcysteine decarboxylase/phosphopantothenate synthase
Cj0828c	71	25	Threonine dehydratase
Cj0842	79	25	Putative lipoprotein
Cj0859c	50	8	Hypothetical protein
Cj0864	100	58	Putative periplasmic protein
Cj0890c	100	67	Putative sensory transduction transcriptional regulator
Cj0894c	100	58	4-hydroxy-3-methylbut-2-enyl diphosphate reductase
Cj0902	93	50	Putative glutamine transport ATP-binding protein
Cj0904c	93	50	Putative RNA methylase
Cj0919c	100	67	Putative ABC-type amino-acid transporter permease protein
Cj0923c	93	25	Putative MCP protein methyltransferase
Cj0938c	93	50	2-acyl-glycerophospho-ethanolamine acyltransferase
Cj0940c	86	25	Putative glutamine transport system permease
Cj0960c	71	25	Ribonuclease P protein component
Cj0965c	86	33	Putative acyl-CoA thioester hydrolase
Cj1012c	100	58	Hypothetical protein
Cj1023c	100	67	Aspartate-semialdehyde dehydrogenase
Cj1051c	100	67	Restriction modification enzyme
Cj1072	57	8	30S ribosomal protein S18
Cj1083c	79	33	Putative nuclease
Cj1085c	79	33	Transcription-repair coupling factor
Cj1089c	86	42	Hypothetical protein
Cj1090c	93	50	Putative lipoprotein
Cj1102	86	42	tRNA pseudouridine synthase B
Cj1112c	64	17	Methionine sulfoxide reductase B
Cj1136	71	17	Putative glycosyltransferase

Cj1139c	57	0	Beta-1,3 galactosyltransferase
Cj1146c	93	42	Putative glucosyltransferase
Cj1151c	100	67	ADP-glyceromanno-heptose 6-epimerase
Cj1152c	79	17	D,D-heptose 1,7-bisphosphate phosphatase
Cj1163c	100	67	Putative cation transport protein
Cj1168c	100	58	Putative integral membrane protein (dedA homolog)
Cj1169c	71	8	Putative periplasmic protein
Cj1172c	100	67	Hypothetical protein
Cj1219c	100	67	Putative periplasmic protein
Cj1219c	79	33	Putative periplasmic protein
Cj1221	100	67	Chaperonin GroEL
Cj1222c	100	67	Two-component sensor (histidine kinase)
Cj1224	93	42	Putative iron-binding protein
Cj1247c	93	33	Hypothetical protein
Cj1251	100	58	Conserved hypothetical protein Cj1251
Cj1298	93	33	Putative N-acetyltransferase
Cj1305c	93	50	Hypothetical protein
Cj1310c	100	67	Hypothetical protein
Cj1318	71	25	Motility accessory factor (function unknown)
Cj1329	86	33	Putative sugar-phosphate nucleotide transferase
Cj1333	57	8	PseD protein
Cj1351	93	50	Phospholipase A
Cj1365c	100	58	Putative secreted serine protease
Cj1386	100	67	Ankyrin-repeat containing protein
Cj1397	57	0	Putative ferrous iron transport protein
Cj1404	86	42	Putative nicotinate-nucleotide adenylyltransferase
Cj1405	64	8	Conserved hypothetical protein Cj1405
Cj1406c	86	42	Putative periplasmic protein
Cj1422c	64	17	Putative sugar transferase
Cj1437c	50	8	Aminotransferase
Cj1442c	71	25	Putative sugar transferase
Cj1443c	93	50	D-arabinose 5-phosphate isomerase
Cj1447c	93	33	Capsule polysaccharide export ATP-binding protein
Cj1448c	71	8	Capsule polysaccharide export system inner membrane protein
Cj1449c	71	17	Hypothetical protein
Cj1450	86	25	Putative ATP/GTP-binding protein
Cj1459	100	58	Hypothetical protein
Cj1477c	100	67	Putative hydrolase
Cj1483c	86	42	Putative lipoprotein
Cj1497c	93	50	Hypothetical protein
Cj1509c	100	67	Putative formate dehydrogenase, cytochrom B subunit

Cj1518	50	8	Putative molybdopterin converting factor,subunit 2
Cj1521c	86	42	Putative CRISPR-associated protein
Cj1560	86	42	Predicted permease
Cj1569c	57	8	NADH dehydrogenase I chain K
Cj1582c	93	50	Putative peptide ABC-transport system permease protein
Cj1616	86	42	Putative haemin uptake system ATP-binding protein
Cj1628	86	42	Putative exbB/tolQ family transport protein
Cj1656c	100	58	Hypothetical protein
Cj1694c	57	0	30S ribosomal protein S14
CJE0051	36	0	Hypothetical protein
CJE0053	36	0	Hypothetical protein
CJE0595	57	8	Hypothetical protein
CJE0598	43	0	Hypothetical protein
CJE0841	64	17	Hypothetical protein
CJE1153	71	25	Hypothetical protein
CJE1418	36	0	Phage integrase family site specific recombinase
CJE1425	36	0	Hypothetical protein
CJE1458	36	0	HK97 family major capsid protein
CJE1461	36	0	Hypothetical protein
CJE1733	36	0	Arsenical-resistance protein, putative
CJJ81176_pTet0051	57	8	Hypothetical protein
CJJCF936_0161	93	33	Hypothetical protein
hypA	79	33	Hydrogenase nickel insertion protein HypA
panC	100	67	Pantoate--beta-alanine ligase
pTet_04	36	0	Hypothetical protein
pTet_16	36	0	Similar to ATPase
pTet_17	50	8	Hypothetical protein
pVir_Cjp44	100	67	Unknown
rpiB	93	42	Ribose 5-phosphate isomerase B
uxaA1	93	33	Putative altronate hydrolase C-terminus

Table C3. Genes present with statistically significant differences between strains from animal and clinical sources. There were 121 genes with significant differences (in presence) between animal and clinical strains. All the genes were more prevalent in animal strains, except two RM1221 hypothetical proteins (CJE0388 and CJE0389). Percentage of strains was obtained by dividing the number of strains in a source carrying the gene with the total number of strains in the source.

Gene	% Present in animal	% present in clinical	Annotation
ald1	43	0	Putative aldehyde dehydrogenase N-terminus
CJE1106	36	0	Hypothetical protein
CJE1117	36	0	Hypothetical protein
CJE1136	36	0	Hypothetical protein
CJE1420	36	0	Hypothetical protein
CJE1421	36	0	Site-specific DNA-methyltransferase
CJE1423	36	0	Hypothetical protein
CJE1426	36	0	Hypothetical protein
CJE1430	36	0	Putative RloG protein
CJE1435	36	0	DNA-binding protein Roi
CJE1453	36	0	Hypothetical protein
CJE1455	36	0	Hypothetical protein
CJE1459	36	0	Hypothetical protein
CJE1460	43	0	Hypothetical protein
CJE1466	36	0	Hypothetical protein
CJE1468	36	0	HK97 family protein
CJE1472	36	0	Putative phage terminase small subunit
CJE1497	36	0	Acetyltransferase
CJE1498	36	0	Formyl transferase domain-containing protein
CJE0388	14	54	Hypothetical protein
CJE0389	14	54	Hypothetical protein
CJE0550	64	0	Putative site-specific DNA-methyltransferase
CJE0567	36	0	Hypothetical protein
CJE0585	36	0	Hypothetical protein
CJE1451	36	0	Hypothetical protein
CJE0592	57	8	Hypothetical protein
CJE0597	36	0	Hypothetical protein
Cj0033	57	15	Putative integral membrane protein
Cj0038c	93	38	Putative poly(A) polymerase family protein
Cj0119	79	31	Putative hydrolase
Cj0133	50	0	Putative glycoprotease family protein
Cj0185c	64	15	Putative phnA domain protein
Cj0232c	57	8	Putative integral membrane protein
Cj0241c	100	69	Putative iron-binding protein
Cj0246c	93	46	Putative MCP-domain signal transduction protein
Cj0271	93	54	Bacterioferritin comigratory protein homolog
Cj0302c	79	31	Putative molybdenum-pterin binding protein
Cj0310c	57	8	Putative efflux protein
Cj0327	50	8	Putative endoribonuclease L-PSP family protein

Cj0370	93	54	30S ribosomal protein S21
Cj0375	50	0	Putative lipoprotein
Cj0424	93	38	Putative acidic periplasmic protein
Cj0465c	93	54	Group III truncated haemoglobin
Cj0480c	93	38	Putative transcriptional regulator
Cj0481	93	46	Putative dihydrodipicolinate synthase
Cj0483	93	31	Putative altronate hydrolase C-terminus
Cj0484	100	62	Putative MFS (Major Facilitator Superfamily) transport protein
Cj0485	86	31	Short chain dehydrogenase
Cj0490	86	31	Putative aldehyde dehydrogenase C-terminus
Cj0496	86	38	Conserved hypothetical protein Cj0496
Cj0520	57	8	Hypothetical protein
Cj0526c	79	31	Flagellar hook-basal body protein FliE
Cj0527c	100	62	Flagellar basal body rod protein FlgC
Cj0594c	71	23	Putative DNA/RNA non-specific endonuclease
Cj0710	86	8	30S ribosomal protein S16
Cj0714	100	69	50S ribosomal protein L19
Cj0741	86	23	Hypothetical protein
Cj0785	100	62	NapD protein homolog
Cj0808c	43	0	Small hydrophobic protein
Cj0815	50	8	Hypothetical protein
Cj0823	57	15	Hypothetical protein
Cj0836	57	15	Methylated-DNA--protein-cysteine methyltransferase
Cj0842	79	31	Putative lipoprotein
Cj0844c	64	0	Putative integral membrane protein
Cj0864	100	69	Putative periplasmic protein
Cj0894c	100	69	4-hydroxy-3-methylbut-2-enyl diphosphate reductase
Cj0943	79	31	Outer-membrane lipoprotein carrier protein
Cj0960c	71	8	Ribonuclease P protein component
Cj0979c	86	46	Putative secreted nuclease
Cj0984	36	0	Conserved hypothetical protein Cj0984
Cj1002c	43	0	Putative phosphoglycerate/bisphosphoglycerate mutase
Cj1047c	43	0	Putative thiamine biosynthesis protein
Cj1113	57	15	Conserved hypothetical protein Cj1113
Cj1136	71	8	Putative glycosyltransferase
Cj1137c	86	38	Putative glycosyltransferase
Cj1139c	57	8	Beta-1,3 galactosyltransferase
Cj1141	50	8	Sialic acid synthase (N-acetylneuraminic acid synthetase)
Cj1148	79	15	Heptosyltransferase II
Cj1152c	79	31	D,D-heptose 1,7-bisphosphate phosphatase

Cj1221	100	69	Chaperonin GroEL
Cj1247c	93	46	Hypothetical protein
Cj1248	36	0	GMP synthase
Cj1251	100	54	Conserved hypothetical protein Cj1251
Cj1313	64	15	N-acetyltransferase specific for PseC product,UDP-4-amino-4,6-dideoxy-beta-L-AltNAc
Cj1324	93	54	Hypothetical protein
Cj1328	100	69	Putative UDP-N-acetylglucosamine 2-epimerase
Cj1384c	71	23	Hypothetical protein
Cj1397	57	8	Putative ferrous iron transport protein
Cj1404	86	38	Putative nicotinate-nucleotide adenylyltransferase
Cj1405	64	15	Conserved hypothetical protein Cj1405
Cj1426c	50	8	Putative methyltransferase family protein
Cj1429c	50	8	Hypothetical protein
Cj1430c	57	8	Putative ferrous iron transport protein
Cj1437c	50	8	Aminotransferase
Cj1440c	93	31	Putative sugar transferase
Cj1464	57	15	Hypothetical protein
Cj1486c	86	46	Putative periplasmic protein
Cj1501	57	8	Hypothetical protein
Cj1555c	93	54	Hypothetical protein
Cj1560	86	46	Predicted permease
Cj1569c	50	8	NADH dehydrogenase I chain K
Cj1623	100	62	Hypothetical protein
Cj1642	86	31	Hypothetical protein
Cj1702c	100	62	50S ribosomal protein L22
CJE0053	36	0	Hypothetical protein
CJE0594	43	0	Hypothetical protein
CJE0598	43	0	Hypothetical protein
CJE1093	50	0	Hypothetical protein
CJE1110	36	0	Hypothetical protein
CJE1151	36	0	Hypothetical protein
CJE1153	71	0	Hypothetical protein
CJE1418	36	0	Phage integrase family site specific recombinase
CJE1425	36	0	Hypothetical protein
CJE1458	36	0	HK97 family major capsid protein
CJE1461	36	0	Hypothetical protein
CJE1471	43	0	Phage terminase, large subunit, putative
CJJ81176_pTet0051	57	8	Hypothetical protein
CJJCF936_1133	93	46	Conserved hypothetical protein
exbD3	93	54	Putative exbD/tolR family transport protein
hypA	79	23	Hydrogenase nickel insertion protein HypA
uxaA1	93	38	Putative altronate hydrolase C-terminus

Figure C1. Focus on presence of genes encoded by *Campylobacter jejuni* integrated elements. The presence of CJIE1 (Pink), CJIE2 (Brown), CJIE3 (Orange), CJI4 (Purple) in clinical (Green), animal (Yellow) and water (Blue) strains wad determined by microarray analysis. Results for genes and arrays were normalized. Using the Cluster 3.0 program (Human Genome Center, University of Tokyo), strains and genes were clustered according to the Pearson's correlation (uncentered) similarity metric. The hierarchical clustering map illustrates high (Green) and low (Red) levels of gene presence as well as absence of genes (Black).

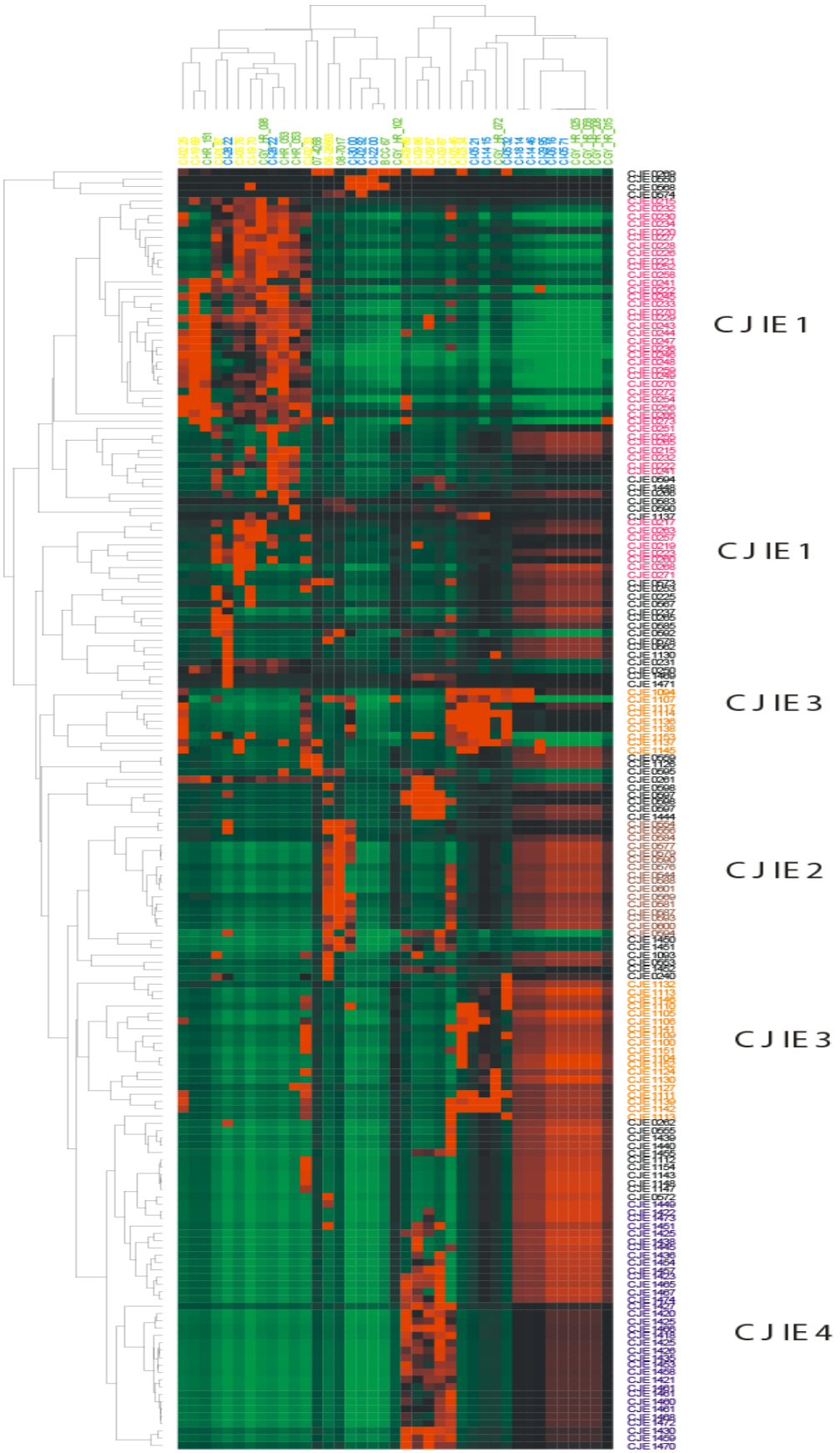

CJIE1
CJIE1
CJIE3
CJIE2
CJIE3
CJIE4

Table C4. Phage genes in *Campylobacter* clinical, animal and water isolates. The presence of genes from CJIE1, CJIE2, CJIE3 and CJIE4 insertions was determined by microarray for 14 animal, 13 clinical and 12 water strains. CJIE1 encodes a Mu-like prophage 1 while CJIE2 and CJIE4 express phage-related genes. CJIE3 is an integrated plasmid. The number and proportions of strains carrying genes of these insertions was calculated for the source groups individually and as a whole.

	Quantity of strains carrying genes in insertions			
Sources	CJIE1	CJIE2	CJIE3	CJIE4
Animal	11 (79%)	10 (72%)	11 (79%)	7 (50%)
Clinical	8 (62%)	5 (38%)	6 (46%)	2 (15%)
Water	5 (42%)	5 (42%)	8 (67%)	3 (25%)
Total	24 (62%)	20 (51%)	25 (64%)	12 (31%)

Table C5. Genes with statistically significant differences in expression between clinical and water isolates. Twenty-five genes had differential expression between clinical and water strains. Genes *Cj0264c*, *Cj0437*, *Cj0196c*, *Cj0203* were more prevalent in water strains while the rest of the genes had a higher number of clinical strains expressing them. Percentage of strains was obtained by dividing the number of strains in a source expressing the gene with the total number of strains in the source.

Gene	% Present in Clinical	% Present in Water	Annotation
CJE0266	33	0	Hypothetical protein
CJE0550	50	0	Putative site-specific DNA-methyltransferase
Cj0011c	75	31	Putative non-specific DNA binding protein
Cj0196c	42	85	Amidophosphoribosyltransferase
Cj0203	25	69	Putative citrate transporter
Cj0264c	42	100	Molybdopterin containing oxidoreductase
Cj0437	50	100	Succinate dehydrogenase flavoprotein subunit
Cj0510c	75	31	Conserved hypothetical protein Cj0510c
Cj0527c	83	23	Flagellar basal body rod protein FlgC
Cj0528c	58	15	Flagellar basal body rod protein FlgB
Cj0702	67	23	Phosphoribosylaminoimidazole carboxylase catalytic subunit
Cj1020c	58	15	Putative cytochrome C
Cj1087c	75	31	Putative peptidase
Cj1242	58	15	Hypothetical protein
Cj1486c	58	15	Putative periplasmic protein
Cj1495c	75	31	Conserved hypothetical protein Cj1495c
Cj1522c	58	15	Putative CRISPR-associated protein
Cj1668c	58	0	Putative periplasmic protein
Cj1715	50	0	Putative acetyltransferase
CJJCF936_1133	67	23	Conserved hypothetical protein
clpP	100	62	ATP-dependent Clp protease proteolytic subunit
NeuA1	75	23	Two-domain bifunctional protein (beta-1,4-N-acetylgalactosaminyltransferase/CMP-Neu5Ac synthase)
hydD	92	38	Putative hydrogenase maturation protease
pabA	58	8	Para-aminobenzoate synthase glutamine Amidotransferase, component II
pVir_Cjp40	75	31	Unknown

Table C6. Genes with statistically significant differences in expression between animal and water isolates. One hundred seventeen genes had differential expression between the animal and water strains. All genes were more prevalent in animal strains. The percentage of strains was obtained by dividing the number of strains in a source expressing the gene with the total number of strains in the source.

Gene	% Present in animal	% Present in water	Annotation
aroA	100	67	3-phosphoshikimate 1-carboxyvinyltransferase
atpC	100	67	ATP synthase subunit epsilon
atpF2	86	42	ATP synthase subunit B
cdsA	79	33	Phosphatidate cytidylyltransferase
Cj0028	86	42	Putative single-stranded-DNA-specific exonuclease
Cj0079c	64	17	Cytolethal-distending toxin A
Cj0081	93	33	Cytochrome bd oxidase subunit I
Cj0085c	64	8	Putative amino acid recemase
Cj0109	50	8	Putative MotA/TolQ/ExbB proton channel family protein
Cj0142c	64	17	Putative ABC transporter ATP-binding protein
Cj0150c	100	58	Aspartate aminotransferase
Cj0166	71	17	tRNA delta(2)-isopentenylpyrophosphate transferase
Cj0174c	71	25	Putative iron-uptake ABC transport system permease protein
Cj0244	86	42	50S ribosomal protein L35
Cj0265c	71	25	Putative cytochrome C-type haem-binding periplasmic protein
Cj0266c	50	8	Putative integral membrane protein
Cj0274	100	42	UDP-N-acetylglucosamine acyltransferase
Cj0288c	79	25	Lipid-A-disaccharide synthase
Cj0318	79	33	Flagellar MS-ring protein
Cj0321	64	17	1-deoxy-D-xylulose-5-phosphate synthase
Cj0353c	86	42	Phosphatase
Cj0369c	79	33	Putative ferredoxin domain-containing integral membrane protein
Cj0371	100	58	Hypothetical protein
Cj0386	86	33	GTP-binding protein EngA
Cj0399	100	58	Colicin V production protein homolog
Cj0404	100	67	Putative transmembrane protein
Cj0432c	43	0	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase
Cj0436	36	0	Putative pyridoxamine 5'-phosphate oxidase
Cj0447	71	25	Putative NUDIX hydrolase family protein
Cj0463	79	33	Zinc protease-like protein
Cj0468	79	17	Amino-acid ABC transporter integral membrane protein
Cj0481	64	17	Putative dihydrodipicolinate synthase
Cj0484	57	8	Putative MFS (Major Facilitator Superfamily) transport protein
Cj0486	64	8	Fucose permease [Carbohydrate transport and metabolism]
Cj0490	43	0	Putative aldehyde dehydrogenase C-terminus
Cj0497	79	8	Putative lipoprotein
Cj0507	57	8	Maf-like protein

Cj0515	79	33	Putative periplasmic protein
Cj0546	79	33	Putative 3-octaprenyl-4-hydroxybenzoate carboxy-lyase
Cj0556	79	33	Putative amidohydrolase family protein
Cj0569	100	67	Hypothetical protein
Cj0633	71	25	Putative periplasmic protein
Cj0646	79	25	Putative lipoprotein
Cj0653c	100	67	Putative aminopeptidase
Cj0659c	71	25	Putative periplasmic protein
Cj0701	93	33	Putative protease
Cj0706	71	25	Conserved hypothetical protein Cj0706
Cj0710	50	8	30S ribosomal protein S16
Cj0713	93	42	tRNA (guanine-N(1)-)-methyltransferase
Cj0723c	100	67	Putative integral membrane zinc-metalloprotease
Cj0732	79	25	ABC transport system ATP-binding protein
Cj0774c	86	42	ABC transport system ATP-binding protein
Cj0777	100	58	Putative ATP-dependent DNA helicase
Cj0784	79	33	Putative periplasmic protein
Cj0801	86	33	Putative integral membrane protein (MviN homolog)
Cj0802	100	67	Cysteinyl-tRNA synthetase
Cj0804	79	33	Dihydroorotate dehydrogenase 2
Cj0824	79	17	Undecaprenyl diphosphate synthase
Cj0883c	57	8	Putative transcriptional regulator
Cj0898	93	50	Putative histidine triad (HIT) family protein
Cj0905c	50	8	Alanine racemase
Cj0919c	71	25	Putative ABC-type amino-acid transporter permease protein
Cj0923c	36	0	Putative MCP protein methyltransferase
Cj0934c	100	58	Putative sodium:amino-acid symporter family protein
Cj0946	93	50	Putative lipoprotein
Cj0950c	100	67	Putative lipoprotein
Cj0953c	86	25	Bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase
Cj0958c	86	25	Putative inner membrane protein translocase component YidC
Cj0958c	86	42	Putative inner membrane protein translocase component YidC
Cj0977	50	8	Hypothetical protein
Cj1005c	79	33	Putative membrane bound ATPase
Cj1007c	100	67	Putative mechanosensitive ion channel family protein
Cj1013c	100	17	Putative cytochrome C biogenesis protein
Cj1045c	100	67	Thiazole synthase
Cj1046c	86	33	Thiamine biosynthesis protein ThiF
Cj1125c	50	8	GalNAc transferase
Cj1127c	79	33	GalNAc transferase
Cj1150c	71	17	D-beta-D-heptose 7-phosphate kinase/D-beta-D-heptose 1-Phosphate adenylyltransferase

Cj1175c	100	58	Arginyl-tRNA synthetase
Cj1192	93	50	Putative C4-dicarboxylate transport protein
Cj1195c	57	8	Dihydroorotase
Cj1206c	100	67	Putative signal recognition particle protein
Cj1214c	86	17	Putative exporting protein
Cj1219c	71	25	Putative periplasmic protein
Cj1226c	86	42	Putative two-component sensor (histidine kinase)
Cj1275c	71	25	Putative peptidase M23 family protein
Cj1286c	86	42	Uracil phosphoribosyltransferase
Cj1303	71	25	3-oxoacyl-(acyl carrier protein) synthase III
Cj1324	86	17	Hypothetical protein
Cj1365c	86	25	Putative secreted serine protease
Cj1376	36	0	Putative periplasmic protein
Cj1398	50	8	Ferrous iron transport protein
Cj1429c	50	8	Hypothetical protein
Cj1437c	43	0	Aminotransferase
Cj1445c	64	17	Capsule polysaccharide export system inner membrane protein
Cj1450	43	0	Putative ATP/GTP-binding protein
Cj1454c	79	33	Putative radical SAM domain family protein
Cj1491c	50	8	Putative two-component regulator
Cj1503c	86	42	Putative proline dehydrogenase/delta-1-pyrroline-5-carboxylate dehydrogenase
Cj1509c	93	50	Putative formate dehydrogenase, cytochrom B subunit
Cj1516	100	58	Putative periplasmic oxidoreductase
Cj1593	100	67	30S ribosomal protein S11
Cj1638	93	50	DNA primase
Cj1654c	50	8	Na(+)/H(+) antiporter
Cj1658	50	8	Putative iron permease
Cj1691c	100	67	50S ribosomal protein L18
Cj1699c	64	17	50S ribosomal protein L29
Cj1725	93	25	Putative periplasmic protein
cynT	100	67	Carbonic anyhydrase
folD	86	42	Bifunctional protein, oxidation and hydrolysis
gyrB	100	50	DNA gyrase B subunit
hemA	79	33	Glutamyl-tRNA reductase
ileS	100	67	Isoleucyl-tRNA synthetase
nrdA	93	42	Ribonucleoside-diphosphate reductase subunit alpha
panC	100	50	Pantoate--beta-alanine ligase
pgi	100	58	Putative glucose-6-phosphate isomerase
rpoB	93	50	DNA-directed RNA polymerase subunit beta
trpB	100	67	Tryptophan synthase subunit beta

Table C7. Genes with statistically significant differences in expression between strains from animal and clinical sources. All of the 227 genes with differential expression between clinical and animal strains were more prevalent in animal strains. Percentage of strains was obtained by dividing the number of strains in a source expressing the gene with the total number of strains in the source.

Gene	% Present in animal	% Present in clinical	Annotation
81116_orf28e	86	46	Unknown
aroA	100	62	3-phosphoshikimate 1-carboxyvinyltransferase
atpC	100	54	ATP synthase subunit epsilon
atpF2	86	38	ATP synthase subunit B
atpG	100	69	ATP synthase subunit gamma
bioA	64	15	Adenosylmethionine-8-amino-7-oxononanoate transaminase
bioB	86	38	Putative biotin synthase
carB	100	62	Carbamoyl-phosphate synthase large subunit
cbpA	93	54	Co-chaperone protein DnaJ
CJE0269	79	23	Putative bacteriophage DNA transposition protein B
Cj0013	100	69	Dihydroxy-acid dehydratase
Cj0028	86	38	Putative single-stranded-DNA-specific exonuclease
Cj0035c	71	23	Putative efflux protein
Cj0041	86	46	Putative flagellar hook-length control protein
Cj0045c	57	15	Putative iron-binding protein
Cj0063c	79	15	Putative ATP-binding protein
Cj0073c	100	69	Conserved hypothetical protein Cj0073c
Cj0081	93	31	Cytochrome bd oxidase subunit I
Cj0100	100	62	ParA family protein
Cj0110	57	15	Putative exbD/tolR family transport protein
Cj0131	100	69	Putative peptidase M23 family protein
Cj0142c	64	8	Putative ABC transporter ATP-binding protein
Cj0166	71	23	tRNA delta(2)-isopentenylpyrophosphate transferase
Cj0232c	36	0	Putative integral membrane protein
Cj0233c	93	38	Orotate phosphoribosyltransferase
Cj0241c	100	54	Putative iron-binding protein
Cj0248	100	69	Tryptophan synthase subunit beta
Cj0255c	86	31	Exodeoxyribonuclease
Cj0266c	50	8	Putative integral membrane protein
Cj0267c	79	31	Putative integral membrane protein
Cj0274	100	54	UDP-N-acetylglucosamine acyltransferase
Cj0282c	100	62	Putative phosphoserine phosphatase
Cj0288c	79	31	Lipid-A-disaccharide synthase
Cj0296c	86	38	Aspartate alpha-decarboxylase
Cj0312	93	38	Peptidyl-tRNA hydrolase
Cj0314	64	15	Diaminopimelate decarboxylase
Cj0316	57	15	Chorismate mutase/prephenate dehydratase
Cj0319	86	31	Flagellar motor switch protein G

Cj0332c	100	69	Nucleoside diphosphate kinase
Cj0337c	79	31	Flagellar motor protein MotA
Cj0339	93	54	Putative MFS (Major Facilitator Superfamily) transport protein
Cj0342c	86	46	Excinuclease ABC subunit A
Cj0371	100	54	Hypothetical protein
Cj0381c	93	38	Orotidine 5'-phosphate decarboxylase
Cj0383c	86	31	6,7-dimethyl-8-ribityllumazine synthase
Cj0386	86	38	GTP-binding protein EngA
Cj0390	100	69	Putative transmembrane protein
Cj0399	100	38	Colicin V production protein homolog
Cj0419	36	0	Putative histidine triad (HIT) family protein
Cj0421c	36	0	Putative integral membrane protein
Cj0436	36	0	Putative pyridoxamine 5'-phosphate oxidase
Cj0452	57	15	DNA polymerase III subunit epsilon
Cj0454c	79	31	Hypothetical protein
Cj0459c	86	38	Hypothetical protein
Cj0463	79	31	Zinc protease-like protein
Cj0467	57	15	Amino-acid ABC transporter integral membrane protein
Cj0468	79	8	Amino-acid ABC transporter integral membrane protein
Cj0481	64	15	Putative dihydrodipicolinate synthase
Cj0486	64	8	Fucose permease [Carbohydrate transport and metabolism]
Cj0497	79	15	Putative lipoprotein
Cj0500	93	46	tRNA 2-selenouridine synthase
Cj0505c	100	69	Putative aminotransferase (degT family)
Cj0507	57	15	Maf-like protein
Cj0510c	79	31	Conserved hypothetical protein Cj0510c
Cj0511	100	69	Putative secreted protease
Cj0527c	71	23	Flagellar basal body rod protein FlgC
Cj0528c	57	15	Flagellar basal body rod protein FlgB
Cj0529c	79	31	Putative aminodeoxychorismate lyase family protein
Cj0553	100	62	Putative integral membrane protein
Cj0557c	79	23	Putative integral membrane protein
Cj0558c	100	62	Gamma-glutamyl phosphate reductase
Cj0562	86	38	Replicative DNA helicase
Cj0569	100	54	Hypothetical protein
Cj0586	86	46	NAD-dependent DNA ligase LigA
Cj0598	36	0	Hypothetical protein
Cj0607	93	54	ABC-type transmembrane transport protein
Cj0633	71	23	Putative periplasmic protein
Cj0639c	93	46	Adenylate kinase

Cj0646	79	31	Putative lipoprotein
Cj0680c	86	46	Excinuclease ABC subunit B
Cj0687c	86	46	Flagellar basal body L-ring protein
Cj0694	100	69	Putative periplasmic protein
Cj0701	93	38	Putative protease
Cj0702	86	23	Phosphoribosylaminoimidazole carboxylase catalytic subunit
Cj0710	50	8	30S ribosomal protein S16
Cj0714	100	54	50S ribosomal protein L19
Cj0732	79	31	ABC transport system ATP-binding protein
Cj0764c	93	54	Arginine decarboxylase
Cj0767c	57	15	Phosphopantetheine adenylyltransferase
Cj0774c	86	31	ABC transport system ATP-binding protein
Cj0775c	100	69	Valyl-tRNA synthetase
Cj0777	100	69	Putative ATP-dependent DNA helicase
Cj0798c	100	69	D-alanyl-alanine synthetase A
Cj0801	86	23	Putative integral membrane protein (MviN homolog)
Cj0802	100	62	Cysteinyl-tRNA synthetase
Cj0817	100	69	Putative glutamine binding periplasmic protein
Cj0824	79	31	Undecaprenyl diphosphate synthase
Cj0843c	100	69	Putative secreted transglycosylase
Cj0873c	100	46	Hypothetical protein
Cj0882c	71	23	Flagellar biosynthesis protein FlhA
Cj0883c	57	15	Putative transcriptional regulator
Cj0887c	100	69	Flagellar hook-associated protein FlgL
Cj0892c	86	38	Putative periplasmic protein
Cj0905c	50	8	Alanine racemase
Cj0914c	100	69	CiaB protein
Cj0941c	36	0	Putative permease
Cj0958c	86	31	Putative inner membrane protein translocase component YidC
Cj0990c	100	69	Hypothetical protein
Cj1007c	100	46	Putative mechanosensitive ion channel family protein
Cj1013c	100	46	Putative cytochrome C biogenesis protein
Cj1020c	64	15	Putative cytochrome C
Cj1031	71	23	Outer membrane component of efflux system (multidrug efflux system CmeDEF)
Cj1032	57	15	Membrane fusion component of efflux system (mutlidrug efflux system CmeDEF)
Cj1034c	100	54	Adenylosuccinate lyase
Cj1046c	86	31	Thiamine biosynthesis protein ThiF
Cj1048c	64	8	Succinyl-diaminopimelate desuccinylase
Cj1054c	71	23	UDP-N-acetylmuramate--L-alanine ligase
Cj1068	57	15	Putative peptidase M50 family protein

Cj1071	100	69	Single-stranded DNA-binding protein
Cj1076	100	54	Pyrroline-5-carboxylate reductase
Cj1087c	79	31	Putative peptidase
Cj1094c	64	15	Preprotein translocase subunit YajC
Cj1097	79	31	Serine/threonine transporter SstT
Cj1106	86	38	Putative periplasmic thioredoxin
Cj1117c	57	15	Ribosomal protein L11 methyltransferase
Cj1119c	100	69	Putative integral membrane protein
Cj1126c	93	54	Oligosaccharide transferase to N-glycosylate proteins
Cj1127c	79	31	GalNAc transferase
Cj1148	79	15	Heptosyltransferase II
Cj1150c	71	23	D-beta-D-heptose 7-phosphate kinase/D-beta-D-Heptose 1-phosphate adenylyltransferase
Cj1157	36	0	DNA polymerase III subunits gamma and tau
Cj1161c	100	69	Putative cation-transporting ATPase
Cj1168c	64	15	Putative integral membrane protein (dedA homolog)
Cj1191c	86	46	Putative PAS domain containing signal-transduction sensor protein
Cj1193c	79	31	Putative periplasmic protein
Cj1207c	100	69	Putative lipoprotein thiredoxin
Cj1214c	86	31	Putative exporting protein
Cj1219c	36	0	Putative periplasmic protein
Cj1235	93	31	Putative peptidase M23 family protein
Cj1237c	64	15	Putative phosphatase
Cj1245c	79	31	Hypothetical protein
Cj1253	93	46	Polynucleotide phosphorylase/polyadenylase
Cj1261	86	46	Two-component regulator
Cj1268c	43	0	5-methylaminomethyl-2-thiouridine methyltransferase
Cj1272c	93	54	Putative guanosine-3',5'-bis(diphosphate) 3'-pyrophosphohydrolase
Cj1286c	86	38	Uracil phosphoribosyltransferase
Cj1292	93	54	Deoxycytidine triphosphate deaminase
Cj1303	71	23	3-oxoacyl-(acyl carrier protein) synthase III
Cj1312	57	15	nucleotidase specific for PseC product,UDP-4-amino-4,6-dideoxy-beta-L-AltNAc
Cj1324	86	46	Hypothetical protein
Cj1328	79	31	Putative UDP-N-acetylglucosamine 2-epimerase
Cj1376	36	0	Putative periplasmic protein
Cj1380	100	69	Putative periplasmic protein
Cj1411c	100	54	Putative cytochrome P450
Cj1426c	50	8	Putative methyltransferase family protein
Cj1429c	50	8	Hypothetical protein
Cj1440c	79	23	Putative sugar transferase
Cj1441c	79	23	UDP-glucose 6-dehydrogenase

Cj1445c	64	15	Capsule polysaccharide export system inner membrane protein
Cj1452	57	8	Putative integral membrane protein
Cj1458c	36	0	Thiamine monophosphate kinase
Cj1484c	79	31	Hypothetical protein
Cj1486c	64	15	Putative periplasmic protein
Cj1493c	64	15	Putative integral membrane protein
Cj1495c	93	31	Conserved hypothetical protein Cj1495c
Cj1504c	50	8	Putative selenide,water dikinase
Cj1515c	86	46	Putative decarboxylase
Cj1522c	71	15	Putative CRISPR-associated protein
Cj1538c	71	23	Putative anion-uptake ABC-transport system ATP-binding protein
Cj1593	100	62	30S ribosomal protein S11
Cj1606c	100	69	Putative ATP/GTP-binding protein (Mrp protein homolog)
Cj1612	71	23	Peptide chain release factor 1
Cj1623	36	0	Hypothetical protein
Cj1665	50	8	Putative lipoprotein thiredoxin
Cj1666c	71	23	Putative periplasmic protein
Cj1680c	79	31	Putative periplasmic protein
Cj1691c	100	69	50S ribosomal protein L18
Cj1698c	100	69	30S ribosomal protein S17
Cj1702c	100	54	50S ribosomal protein L22
Cj1715	43	0	Putative acetyltransferase
Cj1720	57	15	Hypothetical protein
Cj1725	93	38	Putative periplasmic protein
Cj1726c	57	15	Homoserine O-succinyltransferase
CJJ81176_pTet0051	36	0	Hypothetical protein
CJJCF936_0161	43	0	Hypothetical protein
CJJCF936_1133	93	23	Conserved hypothetical protein
clpA	57	15	ATP-dependent Clp protease ATP-binding subunit
clpP	100	62	ATP-dependent Clp protease proteolytic subunit
cysE	57	15	Serine acetyltransferase
exbD1	64	15	Biopolymer ExbD/TolR family transporter
fabZ	86	46	(3R)-hydroxymyristoyl-ACP dehydratase
fliY	100	69	Flagellar motor switch protein FliY
folD	86	46	Bifunctional protein, oxidation and hydrolysis
folE	86	38	GTP cyclohydrolase I
NeuA1	79	23	Two-domain bifunctional protein (beta-1,4-N-acetylgalactosaminyltransferase/CMP-Neu5Ac synthase)
gyrB	100	46	DNA gyrase B subunit
hemC	86	46	Porphobilinogen deaminase
hisA	93	54	phosphoribosylformimino-5-aminoimidazole Carboxamide ribotide isomerase

hisB	100	69	Imidazole glycerol-phosphate dehydratase/histidinol phosphatase
hisC	79	31	Histidinol-phosphate aminotransferase
hisS	79	31	Histidyl-tRNA synthetase
hydD	93	38	Putative hydrogenase maturation protease
hypE	100	54	Hydrogenase expression/formation protein HypE
ktrA	64	15	Putative K+ uptake protein
leuC	100	54	3-isopropylmalate dehydratase large subunit
lon	100	69	ATP-dependent protease La
moaC	86	46	Molybdenum cofactor biosynthesis protein C
msbA	100	62	Lipid export ABC transport protein
murG	79	31	Undecaprenyldiphospho-muramoylpentapeptide beta-N-acetylglucosaminyltransferase
mutS	57	15	Recombination and DNA strand exchange inhibitor
napB	100	69	Periplasmic nitrate reductase small subunit (cytochrome C-type protein)
nrdA	93	46	Ribonucleoside-diphosphate reductase subunit alpha
nuoG	100	69	NADH dehydrogenase gamma subunit
p19	93	54	Periplasmic protein p19
pabA	50	8	Para-aminobenzoate synthase glutamine amidotransferase, component II
pcm	93	46	Protein-L-isoaspartate O-methyltransferase
pgi	100	69	Putative glucose-6-phosphate isomerase
pheT	93	54	Phenylalanyl-tRNA synthetase subunit beta
pTet_36	43	0	Hypothetical protein
pTet_37	36	0	Similar to replication protein Rep
pVir_Cjp34	71	23	Unknown
rpiB	57	15	Ribose 5-phosphate isomerase B
rplO	100	69	50S ribosomal protein L15
rpoB	93	54	DNA-directed RNA polymerase subunit beta
rpsS	93	54	30S ribosomal protein S19
trpB	100	62	Tryptophan synthase subunit beta
wlaH	50	8	Putative galactosyltransferase

Table C8. Up-regulated expression of genes in strains from clinical, animal and water sources. Microarray data was median normalized for both the RNA and the DNA channel. The relative abundance of transcripts was determined for every gene by dividing the normalized value of expression (RNA) by the value of presence (DNA). A median value for the relative abundance of every gene was obtained from the RNA/DNA ratio values of strains in the same source group (Clinical, animal, water). Fold change was calculated by comparing median values for relative abundance of transcripts for the three source groups. Genes were considered up-regulated if they were significantly different using the T-test (p-value <0.01) and at least two fold differences between the source groups were observed. Two genes were up-regulated in clinical strains when compared to water isolates. Strains in the animal source group had 18 genes with higher abundance in transcripts compared to isolates in the clinical source group and 58 genes when compared to the water source group.

Up-regulated genes			
Gene	Gene function	Fold change	T-test
CInical VS Water			
Cj0715	Transthyretin-like periplasmic protein	4.20	1.9E-03
Cj0716	Phospho-2-dehydro-3-deoxyheptonate aldolase	3.08	7.8E-03
Animal VS CInical			
Cj0280	Hypothetical protein	17.71	2.7E-03
Cj1089c	Hypothetical protein	9.88	6.4E-03
Cj0362	Integral membrane protein	8.61	7.1E-03
Cj1486c	Hypothetical protein	7.05	3.7E-04
Cj0480c	Transcriptional regulator	6.52	3.4E-03
NadD (Cj1404)	Nicotinate-nucleotide adenylyltransferase	4.42	5.3E-06
Cj0038c	Poly(A) polymerase family protein	4.22	2.7E-03
Cj1388	Endoribonuclease L-PSP	4.07	7.7E-03
Cj1501	Hypothetical protein	3.74	3.6E-03
hypA (Cj0627)	Hydrogenase expression/formation protein	3.68	3.1E-03
CJE1153	Hypothetical protein	3.21	1.5E-03
Cj1365c	Secreted serine protease	2.74	6.5E-03
Cj0715	Transthyretin-like periplasmic protein	2.50	1.7E-03
Cj1406c	Hypothetical protein	2.34	9.5E-03
CJJCF936_1133	Conserved hypothetical protein	2.24	1.7E-03
Cj0716	Phospho-2-dehydro-3-deoxyheptonate aldolase	2.13	6.8E-03
Cj1491c	Two-component regulator	2.12	9.6E-03
Animal VS Water			
Cj0280	Hypothetical protein	17.71	1.1E-03
pgsA (Cj1067)	CDP-diacylglycerol—glycerol-3-phosphate 3-phosphatidyltransferase	12.14	5.3E-03
Cj1089c	Hypothetical protein	9.88	2.2E-03
Cj0362	Integral membrane protein	8.61	6.1E-04
Cj0044c	Hypothetical protein	8.51	4.9E-03
waaV (Cj1146c)	Glucosyltransferase	7.99	2.7E-03
ald'(Cj0490)	Aldehyde dehydrogenase C-terminus	7.36	7.3E-04
uxaA' (Cj0482)	Altronate hydrolase	6.70	7.1E-04
Cj0480c	Transcriptional regulator	6.52	1.3E-05
Cj0487	Amidohydrolase	6.30	8.6E-03
kpsT (Cj1447c)	Capsule polysaccharide export ATP-binding protein	5.86	2.0E-03
cheR (Cj0923c)	MCP protein methyltransferase	5.62	4.5E-03
Cj1483c	Lipoprotein	5.42	8.4E-03

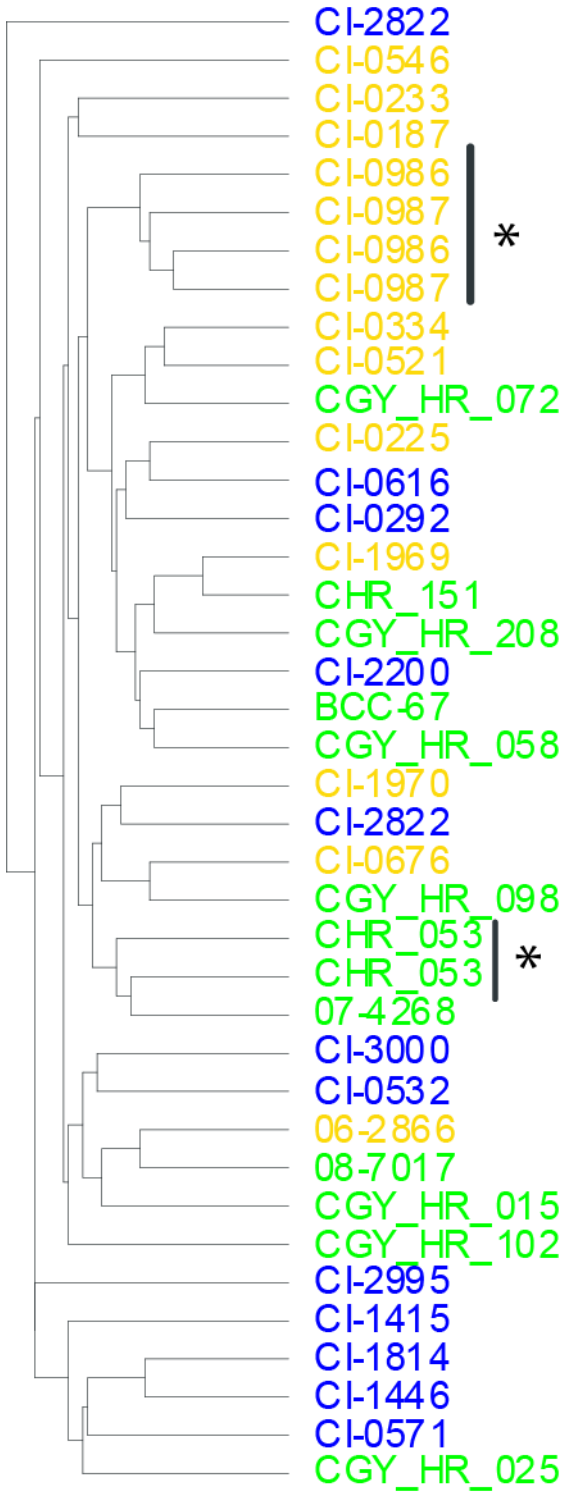
Cj0485	Short chain dehydrogenase	4.98	4.5E-03
dsbB (Cj0865)	Disulfide oxidoreductase	4.78	5.4E-03
bioF (Cj0306c)	8-amino-7-oxononanoate synthase	4.75	1.4E-03
Cj1168c	Integral membrane protein	4.70	3.7E-04
Cj1004	Hypothetical protein	4.42	6.1E-03
Cj1417c	Amidotransferase	4.40	9.3E-03
Cj0429c	Hypothetical protein	4.39	3.3E-03
Cj0038c	Poly(A) polymerase family protein	4.22	7.4E-03
kpsF (Cj1443c)	D-arabinose 5-phosphate isomerase	4.07	3.3E-03
Cj1388	Endoribonuclease L-PSP	4.07	8.0E-03
Cj1251	Hypothetical protein	3.93	3.0E-03
dsbA (Cj0872)	Protein disulfide isomerase	3.91	7.3E-03
recR (Cj1263)	Recombination protein RecR	3.79	8.6E-03
Cj0890c	Sensory transduction transcriptional regulator	3.58	1.8E-03
rplT (Cj0245)	50S ribosomal protein L20	3.56	8.1E-03
hldD (Cj1151c)	ADP-glyceromanno-heptose-6-epimerase	3.53	8.1E-04
Cj1371	Periplasmic protein	3.41	4.8E-03
Cj0152c	Hypothetical protein	3.38	9.3E-03
nadD (Cj1404)	Nicotinate-nucleotide adenylyltransferase	3.29	3.7E-03
Cj0201c	Integral membrane protein	3.22	9.3E-03
murB (Cj1676)	UDP-N-acetylenolpyruvoylglucosamine reductase	3.19	7.7E-03
Cj0062c	Integral membrane protein	3.18	5.9E-03
mrsA (Cj0637c)	Methionine sulfoxide reductase A	3.18	1.8E-03
trmA (Cj0831c)	tRNA (uracil-5-)-methyltransferase	3.11	6.7E-03
Cj0085c	Amino acid recemase	3.09	8.6E-04
rdxA (Cj1066)	Nitroreductase	3.08	2.3E-03
Cj0691	Hypothetical protein	3.04	3.9E-03
Cj0951c	MCP-domain signal transduction protein	2.99	1.1E-03
Cj0440c	Transcriptional regulator	2.86	4.5E-03
Cj0706	Hypothetical protein	2.84	2.1E-03
Cj0484	MFS transport protein	2.81	6.3E-03
exbB3 (Cj0109)	MotA/TolQ/ExbB proton channel family protein	2.79	6.7E-03
Cj1365c	Secreted serine protease	2.74	1.5E-03
cheB' (Cj0924c)	MCP protein-glutamate methylesterase	2.62	6.9E-03
coaD (Cj0767c)	Phosphopantetheine adenylyltransferase	2.60	1.2E-04
cdtA (Cj0079c)	Cytolethal distending toxin A	2.58	4.9E-05
Cj0919c	ABC-type amino-acid transporter permease	2.58	1.6E-03
Cj0645	Secreted transglycosylase	2.47	1.0E-03
Cj0646	Lipoprotein	2.32	6.2E-03

Cj0864	Hypothetical protein	2.26	7.8E-03
Cj0372	Glutathionylspermidine synthase	2.22	2.8E-03
Cj1348c	Coiled-coil protein	2.16	8.8E-03
Cj1111c	MarC family integral membrane protein	2.15	6.4E-03
Cj0054c	Lysine decarboxylase family protein	2.09	6.0E-03
psd (Cj0847)	Phosphatidylserine decarboxylase	2.00	6.8E-03

Table C9. Down-regulated expression of genes in strains from clinical, animal and water sources. Microarray data was median normalized for both the RNA and the DNA channel. The relative abundance of transcripts was determined for every gene by dividing the normalized value of expression (RNA) by the value of presence (DNA). A median value for the relative abundance of every gene was obtained from the RNA/DNA ratio values of strains in the same source group (Clinical, animal, water). Fold change was calculated by comparing median values for relative abundance of transcripts for the three source groups. Genes were considered up-regulated if they were significantly different using the T-test (p-value <0.01) and at least two fold differences between the source groups were observed. Two genes were down-regulated in clinical strains when compared to water isolates. Strains in the animal source group had 1 gene with lower abundance in transcripts compared to isolates in the clinical source group and 5 genes when compared to the water source group.

Down-regulated genes			
Gene	Gene function	Fold change	T-test
Clinical VS Water			
gltB (Cj0007)	Glutamate synthase large subunit	0.49	2.9E-03
Cj1300	SAM domain-containing methyltransferase	0.36	9.0E-03
Animal VS Clinical			
gltB (Cj0007)	Glutamate synthase large subunit	0.33	4.9E-03
Animal VS Water			
flgE (Cj1729c)	Flagellar hook protein FlgE	0.40	9.2E-03
flgD (Cj0042)	Flagellar basal body rod modification protein	0.37	6.4E-03
rrc (Cj0012c)	Non-heme iron protein	0.35	1.6E-04
FlgG2 (Cj0697)	Flagellar basal-body rod protein	0.34	1.9E-03
nrfA (Cj1357c)	Periplasmic cytochrome C	0.31	2.9E-03

Figure C2. Genetic relatedness determined by microarray analysis for strains from clinical, animal and water sources. Relative gene expression (RNA/DNA) was calculated and results were normalized for strains from clinical (Green), animal (Yellow) and water (Blue) sources. Using the Cluster 3.0 program (Human Genome Center, University of Tokyo), strains were clustered according to the Pearson's correlation (uncentered) similarity metric. Clustering of replicate experiments was indicated (*).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CI-0546
CI-0233
CI-0187
CI-0986
CI-0987
CI-0986
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CI-0334
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CGY_HR_072
CI-0225
CI-0616
CI-0292
CI-1969
CHR_151
CGY_HR_208
CI-2200
BCC-67
CGY_HR_058
CI-1970
CI-2822
CI-0676
CGY_HR_098
CHR_053
CHR_053
*
07-4268
CI-3000
CI-0532
06-2866
08-7017
CGY_HR_015
CGY_HR_102
CI-2995
CI-1415
CI-1814
CI-1446
CI-0571
CGY_HR_025

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CONFERENCE POSTERS:

Azzi, G., Carrillo, C.D., Taboada, E.N., Farber, J.M. "*Phenotypic analyses of Campylobacter jejuni Strains isolated from Clinical, Environmental and Animal Sources*". Canadian *Campylobacter* Workshop, October 25-26, 2012, Government Conference Centre, Ottawa, ON, Canada.

Azzi, G., Carrillo, C.D., Taboada, E.N., Farber, J.M. "*Comparative analyses of Campylobacter jejuni Strains isolated from Clinical, Environmental and Animal Sources*". Health Canada Science Forum, November 7-8, 2011, Hampton Inn, Ottawa, ON, Canada.

Azzi, G., Carrillo, C.D., Taboada, E.N., Farber, J.M. "*Comparative analyses of Campylobacter jejuni Strains isolated from Clinical, Environmental and Animal Sources*". International Workshop on *Campylobacter*, *Helicobacter* and Related Organisms (CHRO), August 28-September 1, 2011, Fairmont Hotel Vancouver, Vancouver, BC, Canada.

Azzi, G., Carrillo, C.D., Clark, G.C., Farber, J.M. "*Investigating the Influence of Campylobacter Mu-Like Prophage on Gene Expression in Campylobacter jejuni*". Health Canada Science Forum, November 2-3, 2010, Hampton Inn, Ottawa, ON, Canada.

Azzi, G., Carrillo, C.D., Clark, G.C., Farber, J.M."*Investigating the Influence of Campylobacter Mu-Like Prophage on Gene Expression in Campylobacter jejuni*". BMI Poster Day, May 20, 2010, University of Ottawa, Ottawa, ON, Canada.

Azzi, G., Flint. A., Stintzi, A. "*Investigation* the role of *Cj1000* in *Campylobacter jejuni*". Biochemistry Undergraduate Research Poster Day, April 3, 2009, University of Ottawa, Ottawa, ON, Canada